Published in final edited form as:

J Neurosci Methods. 2012 January 15; 203(1): 146–151. doi:10.1016/j.jneumeth.2011.09.008.

Selective, quantitative measurement of releasable synaptic zinc in human autopsy hippocampal brain tissue from Alzheimer's disease patients

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Abstract

Aberrant central nervous system zinc homeostasis has been reported in Alzheimer's disease (AD). However, there are conflicting reports describing zinc concentration either increased or decreased in the brain of AD patients. Such discrepancies may be due to differences in the brain area examined, zinc detection method, and/or tissue composition. Furthermore, detection and measurement of the releasable zinc pool in autopsy tissue is difficult and usually unreliable. Obtaining an adequate assessment of this releasable zinc pool is of particular significance in AD research in that zinc can coordinate with and stabilize toxic amyloid beta oligomers, which are believed to play a key role in AD neuropathology. In addition, zinc released into the synaptic cleft can interact with the postsynaptic neurons causing altered signaling and synaptic dysfunction, which is a well established event in AD. The method presented here combines two approaches, biochemical fractionation and atomic absorption spectrophotometry, to allow, in addition to extracellular zinc concentration, the reliable and quantitative measurement of zinc specifically localized in synaptic vesicles, which contain the majority of the neuronal releasable zinc. Using this methodology, we found that synaptic vesicle zinc concentrations were increased in AD hippocampi compared to age-matched controls and that this increase in releasable zinc matched increased concentration of zinc in the extracellular space.

Keywords

Zinc, autopsy tissue; synaptic vesicles; Alzheimer's disease; Graphite furnace Atomic absorption spectrophotometry

1. Introduction

Metal levels are altered in Alzheimer's disease (AD) and other neurodegenerative disorders (Lovell, 2009; Watt et al., 2011). It has been demonstrated that the amyloid plaques that

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mark AD neuropathology contain high concentrations of metals such as copper and zinc (Lovell et al., 1998). Remarkably, however, different studies have found that the levels of zinc increase, decrease, or remain unchanged in AD brains as compared to age-matched non demented individuals (Andrasi et al., 1995; Corrigan et al., 1993; Danscher et al., 1997a; Deibel et al., 1996; Panayi et al., 2002; Religa et al., 2006). This discrepancy may be due to differences in the metal analysis technique, brain area examined, and tissue composition (Schrage et al., 2010; Schrage et al., 2011). Indeed, autopsy brain tissue is not homogeneous and difficult to compare across different studies. For example, autopsy brain tissue slices normally collected for frozen inventory and further biochemical analyses, although including comparable brain regions of interest are likely to be from different coronal planes. This would lead to different relative levels of lipids, grey and white matter, blood vessels, and cell types (neurons vs. glia). Total zinc measurements would then be representative of all of these components and thus vary among samples depending on their composition.

Total zinc measurements are a combination of two separate zinc pools in the brain, the bound zinc pool and the releasable or chelatable zinc pool (Frederickson et al., 2000). The bound zinc is incorporated as a cofactor in many proteins such as Cu/Zn superoxide dismutase, insulin degrading enzyme, and several transcription factors (Sandstead et al., 2000). This zinc is bound tightly and is required for protein structure and function. The releasable pool of zinc is quite different in that it is sequestered in pre-synaptic vesicles along with glutamate and can be released as a neurotransmitter upon synaptic stimulation (Assaf and Chung, 1984). Once released in the synaptic cleft, this zinc can modulate postsynaptic receptors and even enter the postsynaptic neuron through specific channels initiating downstream signaling (Bitan et al., 2009; Frederickson et al., 2000). Notably, it has been proposed that this chelatable zinc can interact with amyloid beta oligomers and target them to the postsynaptic density, which would lead to the synaptic dysfunction and cognitive decline that mark the onset and progression of symptomatic AD (Bush et al., 1994; Deshpande et al., 2009; Noy et al., 2008). The possible role of releasable zinc in the development or progression of AD makes the measurement of this pool of zinc particularly important. The measurement of releasable zinc in animal tissue and cultured cells can be accomplished with a variety of methods including histological techniques and fluorescent dyes (Frederickson et al., 1987; Suh et al., 2000). Unfortunately these methods are not optimal or plain inadequate to measure zinc in autopsy brain tissue specimens (Kay, 2006; Schrage et al., 2011). The present report details an alternative method to quantify releasable zinc using a combination of biochemical fractionation and atomic absorption spectrophotometry. The use of this methodology removes the uncertainty of heterogeneous tissue samples by isolation of synaptic vesicles and allows for precise measurement of the zinc contained in these vesicles, thus providing a quantitative assessment of the releasable synaptic zinc.

2. Materials and methods

2.1 Human brain tissue

Frozen mid-hippocampus tissue was obtained from the Oregon Brain Bank at Oregon Health and Science University (OHSU) in Portland, OR. Donor subjects were enrolled and clinically evaluated in studies at the NIH-sponsored Layton Aging and AD Center (ADC) at OHSU. Control subjects were participants in brain aging studies at the ADC. Subjects received annual neurological and neuropsychological evaluation, with clinical dementia rating (CDR) scores assigned by an experienced clinician. Controls had normal cognitive and functional examinations. Tissue use conformed to institutional review board-approved protocols. Brain tissue was examined by a neuropathologist for neurodegenerative pathology including neurofibrillary tangles and neuritic plaques. Using standardized CERAD criteria (Mirra et al., 1991), cases were assigned an amyloid score based on the deposition of

amyloid plaques in the brain (1=severe, 2=moderate, 3=mild, 4=none), and a Braak stage (1–6; with 6 being the most severe) indicative of the level and location of hyper-phosphorylated tau tangles (Braak and Braak, 1991). The cases used in this study are described in Table 1.

2.2 Methods for the analysis of zinc

All laboratory processing was performed under clean contaminant-free conditions, to minimize external metal contamination. Each sample was evaporated to dryness in a drying oven at 80 °C. Digestion of the residue was carried out using 0.5 mL of 30% hydrogen peroxide (GFS Chemicals, Powell, OH) at 70 °C for 18–24 hours followed by 0.10 mL of Ultra-pure nitric acid (GFS Chemicals) until completely ashed (Alcock, 1987). The digested white ash was dissolved in 4.0 mL of Milli-Q deionized distilled water. Each digested sample was further suitably diluted 1:20, 1:40, 1:50, 1:80, 1:100 or 1:200 (v/v) using Milli-Q deionized distilled water prior to analysis.

Concentrations of zinc in the diluted digested samples were determined by graphite furnace-atomic absorption spectrophotometry (GF-AAS). A Varian Instruments Model-240Z Zeeman atomic absorption spectrophotometer (Varian, Inc., Walnut Creek, CA) equipped with a Varian GTA-120 graphite tube atomizer, a PSD-120 programmable sample dispenser, a Varian UltrAA high intensity boosted hollow cathode lamp were routinely used to measure zinc at low parts per billion (ppb) concentrations in solution. Pyrolytically coated Varian graphite partition tubes were used for all analyses. Argon gas (0.3 L/min flow) was used to protect and purge the graphite tubes during the furnace program steps, and the data acquisitions were carried out using a Varian SpectrAA software.

The auto-sampler cups were washed initially with 0.5 N Ultra-pure nitric acid followed by Milli-Q deionized distilled water prior to use. Zinc calibration standards (0.0, 0.5, 1.0, 1.5, and 2.0 microgram per liter (ppb) were prepared in Milli-Q deionized distilled water and run with each set of samples and the absorbance was measured at 213.9 nm wavelength (see Fig. 1A). Milli-Q deionized distilled water was kept and analyzed in between samples to avoid and check any contamination or carryover between samples. Precision and accuracy of the method were routinely checked by digesting known weights of SRM1577a standard liver powder (NIST, Bethesda, MD) and analyzing them routinely along with these samples. These liver powders contained known certified amounts of several metals including zinc.

2.3 Extracellular enriched sample preparation

Two to three hundred mg of frozen mid-hippocampus was homogenized using a 1 ml syringe with a 20 gauge needle in 50 mM Tris-HCl buffer (pH = 7.6) with 0.01% NP-40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged for 5 min at $960 \times g$ at 4°C and the supernatant collected for analysis (Lesne et al., 2006).

2.4 Biochemical fractionation

Subcellular fractionation was performed as described previously (Billa et al., 2010; Phillips et al., 2001). Briefly, hippocampal samples (~0.2 g) were homogenized in buffer containing 0.32 M sucrose, 1 mM MgCl₂ 0.1mM CaCl₂ with protease and phosphatase inhibitors. The homogenate was brought to a final concentration of 1.25 M sucrose by adding 2 M sucrose and 0.1 mM CaCl₂. The homogenate was then placed in an ultracentrifuge tube and overlaid with 1 M sucrose and centrifuged at $100,000 \times g$ for 3 h at 4°C using a Beckman Coulter Optima L-100 XP ultracentrifuge. The synaptosomal fraction was collected at the 1.25 M/1 M sucrose interface, diluted 1:10 with 20 mM Tris-Cl, pH 6, 0.1 mM CaCl₂, containing 1% Triton X-100 (TX-100), mixed and centrifuged at $40,000 \times g$ for 20 min at 4 °C. The

supernatant was collected and concentrated using an Ampicon 50 k cutoff centrifugal filter unit (Millipore, Billerica, MA). The concentrate was then precipitated using acetone overnight at -20°C , centrifuged at $7000 \times g$ for 5 min, and the remaining pellet containing synaptic vesicles was solubilized in 1% SDS.

2.5 Immunoblotting

For immunoblotting, equal amounts of protein as determined using the BCA assay from Pierce (Rockford, IL)) from the various fractions were separated by SDS-PAGE and transferred to a PVDF membrane by electroblotting. The membrane was blocked for one hour with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) then incubated overnight with selective antibodies to synaptophysin (Millipore) and PSD95 (Cell Signaling, Beverly, MA). After incubation with an appropriate fluorescent secondary antibody (1:10,000) (LI-COR Biosciences), the membrane was scanned directly by the Odyssey infrared fluorescent imaging system (LI-COR Biosciences). The blot was then reprobbed with an antibody to GAPDH (Cell Signaling) and imaged again to ensure equal loading and transfer.

2.6 Statistics

Microsoft Excel was used for graph production and SigmaPlot was used for data analysis. Pair-wise comparisons were done using a Student's t-test. Data represents mean $\pm$ SE. Correlation analysis was done using Pearson product moment correlation test.

3. Results

3.1 GF-AAS instrument calibration

A calibration curve was run on the GF-AAS instrument at regular intervals along with the samples to ensure precision and accuracy. Figure 1A shows the calibration curve for zinc standards (0 – 2 ppb).

3.2 Synaptic vesicle isolation

Synaptic vesicles were isolated beginning with the biochemical fractionation method described in the literature using the supernatant taken after the pH = 6 incubation and centrifugation (Phillips et al., 2001). After concentrating the sample, the synaptic vesicle fraction had high protein yields (~ 3 mg/ml in 200 μl). Synaptophysin was used as a synaptic vesicle marker and PSD95, a post-synaptic density marker. The successive steps of the fractionation were probed using Western blot for these markers. The Western blot in Fig. 1B demonstrates the enrichment of synaptophysin and therefore synaptic vesicles using the fractionation protocol.

3.3 Synaptic vesicle zinc levels in AD and age-matched hippocampi

The protein concentration (measured by the BCA protein assay) of the extracellular enriched lysates (500 μg) and the purified synaptic vesicles (250 μg) from control and AD hippocampi were normalized with Milli-Q deionized distilled water. The hydrogen peroxide-nitric acid digestion for GF-AAS was carried out as described in the methods section. The samples were measured for zinc at four different dilutions and each sample was measured twice in this manner. The values for duplicate analyses were not significantly different, demonstrating the precision of the instrument.

The measurements in extracellular enriched fractions showed increased zinc concentration in the AD hippocampi (Fig. 2A), which has been reported previously (Danscher et al., 1997a; Deibel et al., 1996; Religa et al., 2006)). More importantly, the synaptic vesicle zinc

concentration was found to be significantly higher in the AD samples compared to control (Fig. 2B).

3.3 Postmortem interval and biochemical fractionation do not affect synaptic vesicle zinc stores

Figure 3 shows the correlation analysis between zinc concentration measured in extracellular enriched fractions (Fig. 3A) and synaptic vesicles (Fig. 3B) and the postmortem interval (PMI). There was no correlation found with either fraction, suggesting that this method is suitable for autopsy tissue (at least up to 19 hrs – the longest PMI used in the present studies).

To further test the method, we performed a zinc spiking experiment to address the possibility that zinc in the total homogenate (zinc is found in high concentration in amyloid plaques (Stoltenberg et al., 2005)) may contaminate the subsequent fractions and particularly alter the zinc content in the synaptic vesicles during the fractionation procedure. Hippocampal tissue from an aged individual was homogenized and split into two equal aliquots. Forty ng of zinc was added to one of the two aliquots and the fractionation for synaptic vesicles, digestion, and zinc analysis were carried out as described in the methods section. Figure 3C shows the zinc concentration measurements at three different sample dilutions for synaptic vesicles that were added with zinc and those that were not illustrating that there was no difference in the zinc concentration. This result demonstrates that zinc in other fractions does not contaminate the synaptic vesicle fraction.

4. Discussion

The method presented here combines biochemical subcellular fractionation with the precision of GF-AAS to detect zinc present in synaptic vesicles. The zinc concentration was found to be increased in the extracellular enriched and synaptic vesicle fractions isolated from the hippocampus of AD patients as compared to age-matched controls. Furthermore, this approach is particularly amenable to autopsy tissue where traditional techniques such as histological and fluorescence detection of zinc are often unsatisfactory.

The method described here allows for more informative zinc measurements to be made as compared to previous approaches measuring metals in whole tissue pieces or total homogenates. Many metals, such as zinc, act as cofactors bound to various proteins. Zinc is essential for correct function of many proteins such as transcription factors, ion channels, enzymes and receptors (Bitanirwe and Cunningham, 2009). In addition, the expression of metallothioneins, which are important functional zinc-binding proteins (Chung and West, 2004), is tightly regulated by zinc itself (isoforms 1 and 2) (Bitanirwe and Cunningham, 2009) in that the expression of this class of proteins increases when zinc concentration is increased. Consequently, overall increases of the bound zinc pool levels may not be dishomeostatic as bound zinc can trigger mechanisms (e.g., increased expression of metallothioneins) that modulate its own impact/availability. On the other hand, increases in the levels of chelatable (releasable) zinc pool can quickly perturb homeostasis, especially free zinc that is released from synaptic vesicles into the microenvironment of the synaptic cleft during synaptic stimulation. Under these conditions, abnormal zinc interactions with the postsynaptic element can profoundly affect neurotransmitter receptor function and thus alter synaptic transmission (Quinta-Ferreira and Matias, 2005; Weiss et al., 1993). Furthermore, compelling evidence suggests that released free zinc can coordinate with amyloid beta, stabilize the toxic oligomeric form, and promote their pathological targeting of the postsynaptic element (Deshpande et al., 2009; Noy et al., 2008). Therefore, by isolating the synaptic vesicles where the releasable zinc is stored as described here, accurate measurements of the zinc pool most significant to AD neuropathology can be achieved.

Only around 5 % of the zinc in the brain is releasable into the synaptic cleft, and therefore detecting changes in these levels is difficult (Frederickson et al., 2000). Histochemical techniques, such as the various Timm's methods for silver staining, or fluorescent dyes can be utilized to measure this zinc population. However, these methods work optimally only in fresh tissue. Silver staining techniques can be used in autopsy tissue for qualitative detection of which cell type/areas contain chelatable zinc, but cannot measure the concentration (Stoltenberg et al., 2005) and staining quality becomes unsatisfactory as soon as 6 hr postmortem (Danscher et al., 1997b). On the other hand, zinc fluorescent dyes, such as TSQ, are highly sensitive but staining varies substantially depending on the PMI and freezing conditions of the tissue (Suh et al., 1999). This severely limits the number of samples that can be compared as the PMI is one condition that varies substantially within a cohort of autopsy tissue specimens. As shown in figure 3, such limitations is less pressing when using the method described here in that there is no correlation between PMI and variability of vesicular zinc concentration in the different sample tissue. Furthermore, our tests show that there is no apparent contamination of the synaptic vesicles zinc from other zinc pools released in the homogenate during the fractionation procedure. This is of particular importance when working with AD brain samples that contain zinc-enriched amyloid plaques from which substantial amounts of zinc may be released during tissue homogenization/processing.

The increase in zinc concentrations that we observed in the extracellular enriched fractions prepared from the AD hippocampi, which include zinc released from the zinc-rich amyloid plaques (Suh et al., 2000), is consistent with findings previously reported (Danscher et al., 1997a; Deibel et al., 1996; Religa et al., 2006). More significantly, we found consistently higher concentrations of releasable zinc in synaptic vesicle isolated from AD brain tissue. Notably, when comparing AD to age-matched controls, such an increase in zinc in the synaptic vesicles (+178%) was much more pronounced than the increase in zinc levels determined in the extracellular fraction (+45%). This could suggest that during synaptic activity, more zinc may be released in the synaptic cleft in the AD hippocampus and thus negatively impact the post-synaptic element and/or bind soluble amyloid beta within the synaptic space. This collective evidence strongly indicates that zinc regulation is altered in AD and suggests that such dysregulation may play a key role in AD pathology. Indeed, recent studies have highlighted the importance of the maintenance of proper zinc homeostasis and that upsetting the balance of zinc can have profound effects on cognitive integrity. For example, zinc supplementation has been shown to delay memory deficits in the transgenic mouse model of AD, 3xTg-AD, and increases levels of the neurotrophic factor brain-derived growth factor, BDNF (Corona et al., 2010). On the other hand, the zinc transporter 3 (ZnT3) KO mouse, which cannot transport releasable zinc into synaptic vesicles, has significant age-dependent learning and memory deficits (Adlard et al., 2010).

In conclusion, we have here described a method to measure releasable, synaptic vesicle-associated zinc that is quantitative, reproducible, and has minimal sensitivity to factors such as variability of PMI among different samples. This method is particularly useful to studies concerned with determining the pathological significance of brain zinc levels in neurodegenerative conditions such as AD where releasable synaptic zinc has been proposed to play a key role in toxic amyloid beta oligomer formation and their dysfunctional targeting to post-synaptic elements.

Highlights

- Zinc homeostasis is altered in Alzheimer's disease. ► Synaptic vesicles were isolated from Alzheimer's disease (AD) and age-matched frozen hippocampal autopsy tissue.
- Graphite furnace atomic absorption spectrophotometry (GF-AAS) was used to

measure zinc in the synaptic vesicles. ► Synaptic vesicle zinc or chelatable zinc was significantly increased in AD hippocampal samples. ► The combination of biochemical fractionation and GF-AAS allows quantitative analysis of chelatable zinc concentration from autopsy tissue.

Acknowledgments

We would like to thank Randy Woltjer at OHSU for the neuropathological analysis and tissue samples, the Human Nutrition Division, Department of Preventive Medicine and Community Health, UTMB for the use of the GF-AAS instrument, and Patrick Simmons, Atomic Spectroscopy Product Specialist, Agilent Technologies, Inc. for setting up the furnace temperature program steps. This work was supported by grants NIH/NINDS R01NS059901 and Alzheimer's Association IIRG-90755 to G.T. and NIH/NIEHS T32ES007254-20 to N.L.B.

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Fig.1.

Methodology assessment. (A) A representative calibration curve generated by the GF-AAS with absorbance measured at 213.9 nm wavelength. The instrument was calibrated before each sample run. (B) Western blot of each fraction; total homogenate (Hom), synaptosome (Syn), and synaptic vesicle (SV), demonstrating the enrichment of synaptophysin (a synaptic vesicle marker) and absence of PSD95 (a postsynaptic marker) in the synaptic vesicle fraction. GAPDH is used to show equal protein loading.

Fig. 2.

Zinc concentrations are increased in AD extracellular enriched and synaptic vesicle samples. The extracellular enriched fraction (A) had increased zinc compared to age-matched controls ($p < 0.001$). Zinc concentration was also increased in the synaptic vesicle fraction in AD ($p = 0.006$) (B). The measurements were carried out at four different dilutions and this was repeated twice with no significant difference between the runs. (n, Control = 4, AD = 5).

Fig. 3.

The postmortem interval and biochemical fractionation do not affect synaptic vesicle zinc stores. Correlation analysis between the postmortem interval and zinc concentration showed no correlation for either extracellular enriched or synaptic vesicle fractions (A). The graph in (B) shows zinc measurements of synaptic vesicles at 3 dilutions on samples that did and did not have 40 ng extra zinc added (spiked) to the total homogenate. There is no significant difference between control and “spiked” sample zinc concentrations, demonstrating no contamination of the zinc content in the isolated synaptic vesicles from zinc present in previous fractions. Pearson’s product moment correlation analysis (3A, 3B) and student’s t-test (C) show no significant differences.

Table 1

Clinical Summary of Case Tissue

Case #	Diagnosis	Age (yrs)	Sex	PMI ^a (hr)	Braak	Plaque
1	---	80	M	2	1	3
2	---	84.8	M	18	0	3
3	---	85.1	M	12	0	4
4	---	>89	M	4.5	1	4
5	AD	64	M	9.25	6	1
6	AD	87	M	8.75	6	2
7	AD	60	F	4.5	6	2
8	AD	62	F	6.5	6	2
9	AD	82	F	19	6	2

^aPostmortem interval

Table 2

Instrument and Furnace Parameters for the Determination of Zinc

Parameter	Value/Description
Lamp	High intensity boosted Hollow Cathode
Lamp Current (mA)	5
Wavelength (nm)	213.9
Slit width (nm)	1.0
Graphite tube/site	Pyrolytically coated
Matrix Modifier	None
Furnace temperature program Steps	1) 85 °C in 5.0 seconds (s), gas flow (0.3 L/min) 2) 95 °C in 40.0 s, gas flow (0.3 L/min) 3) 120 °C in 10.0 s, gas flow (0.3 L/min) 4) 300 °C in 5.0 s, gas flow (0.3 L/min) 5) 300 °C in 5.0 s, gas flow (0.3 L/min) 6) 300 °C in 2.0 s, no gas flow 7) 1900 °C in 0.8 s, no gas flow (atomization) 8) 1900 °C in 2.0 s, no gas flow 9) 1900 °C in 2.0 s, gas flow (0.3 L/min)