Supplementary methods, figures, and information

Understanding metal homeostasis in primary cultured neurons. Studies using single neuron subcellular and quantitative metallomics.

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Supplementary methods:

Inductively coupled mass spectrometry (ICP-MS): ICP-MS of primary rat cortical mass cultures was done exactly as described previously[1] except that cultures were scraped in chelex treated 26 mM $NaHCO_3$, pH 7.4.

Sample preparation for synchrotron radiation x-ray fluorescence analysis (SRXRF analysis): Neurons attached to SiN windows were fixed by one of two methods: chemical fixation – 8 min in aqueous 4% formaldehyde dissolved in phosphate buffered saline (PBS) and chelex treated, or cryofixation – either manual plunge freezing in 2-methylbutane cooled in liquid N_2 or block freezing by placing the SiN window on dry-ice powder. Chemically fixed neurons were washed three times in PBS (chelex treated) then were dehydrated by air-drying in a desiccated chamber[2] Cryofixed neurons were dehydrated by lyophilization for several hours. All SiN windows were subsequently stored at room temperature under vacuum in a desiccator until SRXRF analysis was performed (within 1 week of fixation). See the methods section of the paper for a detailed description of SRXRF analysis.

Scanning electron microscopy: Post SRXRF scans, dehydrated samples on SiN were mounted on aluminum stubs and coated with a 15 nm layer of 40% (w/w) gold/palladium using a Balzers SCD 050 sputter coater (Oerlikon Balzers, Schaumburg, IL). The coated samples were digitally imaged at 15kV accelerating voltage using a Cambridge S240 Stereoscan scanning electron microscope.

Supplementary results:

Resting steady state metal contents in primary cortical neurons: Using ICP-MS a total resting steady state content for each metal in cultures of primary rat cortical neurons was determined (see supplementary figs. S1A&B). Metal contents obtained from ICP-MS analyses were normalized to total protein concentration in the same sample. For ease of illustration, metals were placed into one of two groups,

either abundant or trace, based on the level obtained by ICP-MS. The rank order of abundant metals in these cultures, based on ICP-MS results was: calcium about 4X zinc; zinc about 2X iron and 5X copper. Zinc levels were 50X or greater when compared to the trace metals chromium, manganese, cobalt, and nickel. Although cortical cultures are populated by several neuronal types, we found the same trends when single cell SRXRF analysis was performed as detailed below.

Comparing two methods of sample preparation on neuronal morphology: SRXRF analysis can be performed on either dehydrated or frozen hydrated samples, although using frozen hydrated samples is technologically more challenging. Using hydrated samples is preferred when metal ion speciation is of interest[3,4]. When using dehydrated samples, good sample preparation is critical for obtaining meaningful and reproducible quantitative SRXRF data. Comparing different methods of sample preparation for SRXRF with values determined by ICP-MS shows that metal levels can be influenced by sample preparation; this is well documented in the literature.[1,5-8] Two methods of fixation were used and compared in this study. The first was chemical fixation followed by brief washing in PBS and dehydration by air drying in a desiccator. The second was cryofixation – either manual plunge freezing in 2-methylbutane cooled in liquid N_2 or block freezing by placing the SiN on dry-ice powder. After cryofixation, the samples were subsequently dehydrated by lyophilization.

Chemical fixation with a PBS wash and air drying results in excellent preservation of cellular morphology and intracellular structure (e.g., see[1,9,10]). Similarly, cryofixation followed by lyophilization yielded well preserved cellular structure even including the fine neuronal processes. This conclusion was arrived at by viewing dehydrated samples pre-labeled with MitoTracker® Green using epifluorescence, or viewing samples with scanning electron microscopy post-SRXRF scanning. Supplementary fig. S2A shows an image of a cryofixed and dehydrated neuron and nearby processes made visible by MitoTracker® Green fluorescence. This neuron was scanned after transporter mediated zinc loading. Fluorescent signals of single intact mitochondria are visible in the processes and the cytoplasm yields the highest level of fluorescence from the overlapping network of mitochondria located there. The nucleus can be differentiated from the rest of the cytoplasm by being relatively devoid of fluorescence. Thus, we can be confident that intracellular structures throughout individual neurons (including the nucleus and soma) are well preserved. Shown in supplementary figs. S2B-E are the corresponding 2-D SRXRF scans obtained from the sample shown in supplementary fig. S2A. Here is shown the subcellular distributions seen for the three most abundant metals found in neurons – calcium, zinc, and iron; and a less abundant metal – manganese. In addition, overall neuronal morphology is well preserved during X-ray exposure, as shown by scanning electron microscopy (SEM, supplementary fig. S2F). The image is representative of neurons and processes visible on the SiN window post-SRXRF scanning[1].

The effect of sample preparation on measured metal levels in single neurons: When total metal contents in single neuron somas obtained with the two different fixation protocols were compared, significant differences for some metals were observed. The data presented in supplementary figs. S3A&B illustrate these differences. The data of supplementary figs. S3A&B shows mean metal content using scans obtained from four separate visits to the 2-ID-D beamline at APS and 9 to 15 single neurons (resting, steady state, without experimental treatment) analyzed. It is noteworthy that average ROI size was not affected by the

fixation protocol: 139 ± 12.2 (chemical fixation) vs. 130 ± 14.4 (cryofixation) μm^2 (mean ± S.E.). These data are similar to other reports of chemically fixed cultured neurons [7]. To understand better the effects of sample preparation on measured metal levels, it is helpful to refer to metal level comparisons shown in fig. S1 obtained from ICP-MS from cultures that were neither fixed nor dehydrated. Metal to metal comparisons are similar when comparing relative levels, one metal to another for either ICP-MS analysis or SRXRF scans of neuronal soma, i.e., calcium >> zinc ≥ iron > copper ≥ manganese > nickel >> either cobalt or chromium. Levels of chromium and cobalt in neuronal soma obtained from SRXRF scans were close to background measurements (see methods) and sometimes negative with the background subtracted. Thus, SRXRF values for these two metals are not included in this analysis. Zinc, iron, and manganese showed decreased metal levels after chemical fixation (although only zinc was statistically significant). A loss of metals after chemical fixation would be best explained by the fixation and subsequent washing allowing the escape of "labile" metals. These data show that compared to all the other metals measured in neurons, zinc has one of the highest average concentrations and the largest proportion of "labile" metal and/or metal bound to freely diffusible ligands. It should be noted that a trend for increased copper and nickel after chemical fixation was seen, but in particular for copper was highly variable and neither effect reached statistical significance. This likely occurs as a result of metal contamination in fixative buffers.[8] Calcium showed significantly larger content levels after chemical fixation when compared to cryofixation. Although calcium too could be a contaminant in the fixative buffers, when comparing the relative levels of metals obtained from ICP-MS to those obtained by SRXRF scanning – the chemical fixation calcium levels matched better.

Supplementary Figure Legends:

Supplementary Figure S1: Inductively coupled plasma mass spectrometry (ICP-MS) of primary cortical mass cultures (6 DIV). Total cellular homogenates from mass cultures were analyzed (see Methods) and total protein was determined using the BioRad assay solution with BSA as a standard. Results expressed as metal content (nmoles) divided by total protein (mg). (**A**) Abundant metals. Tukey's multiple comparison posttest was significant ($p < 0.05$) for all data pairs except iron vs. copper. (**B**) Trace metals. Tukey's multiple comparison posttest was significant ($p < 0.05$) for all data pairs except chromium vs. cobalt and manganese vs. nickel. Each bar represents mean ± S.E., two replicates.

Supplementary Figure S2: (A) Representative image of MitoTracker® Green fluorescence in a cryofixed primary cortical culture (4 DIV) and corresponding 2-D SRXRF scans for **(B)** calcium, **(C)** iron, **(D)** zinc, and **(E)** manganese. **(F)** Scanning electron micrograph (SEM), processed post-SRXRF scanning. See supplementary results for additional details. The culture shown here was treated with 30 µM Zn^{2+} for 3 min prior to cryofixation.

Supplementary Figure S3: Effect of sample preparation on average metal levels in single primary cortical neurons. Regions of interest (ROI) were manually drawn around neuronal soma observed in 2-D SRXRF scans using MAPS software. Metal levels are expressed for each ROI as the average value – ng/cm^2. Data presented are the mean ± S.E., n = 9 single neurons, two separate trips to APS beamline – chemical fixation (chemfix); and n = 15 single neurons, three separate trips to APS beamline – cryofixation (cryofix).

Student's t-test was significant for comparison of chemical fixation vs. cryofixation for calcium and zinc only ($p < 0.05$). **(A)** calcium, iron, and zinc, **(B)** manganese, nickel, and copper.

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Figure S1:

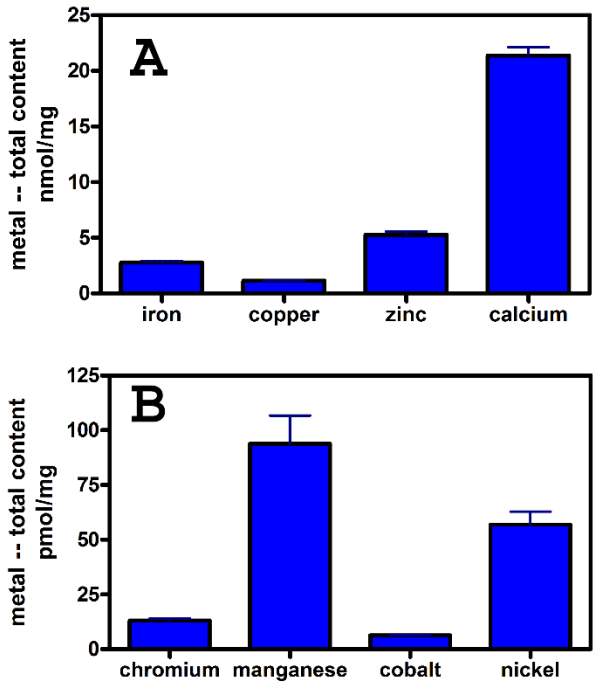
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Figure S2:

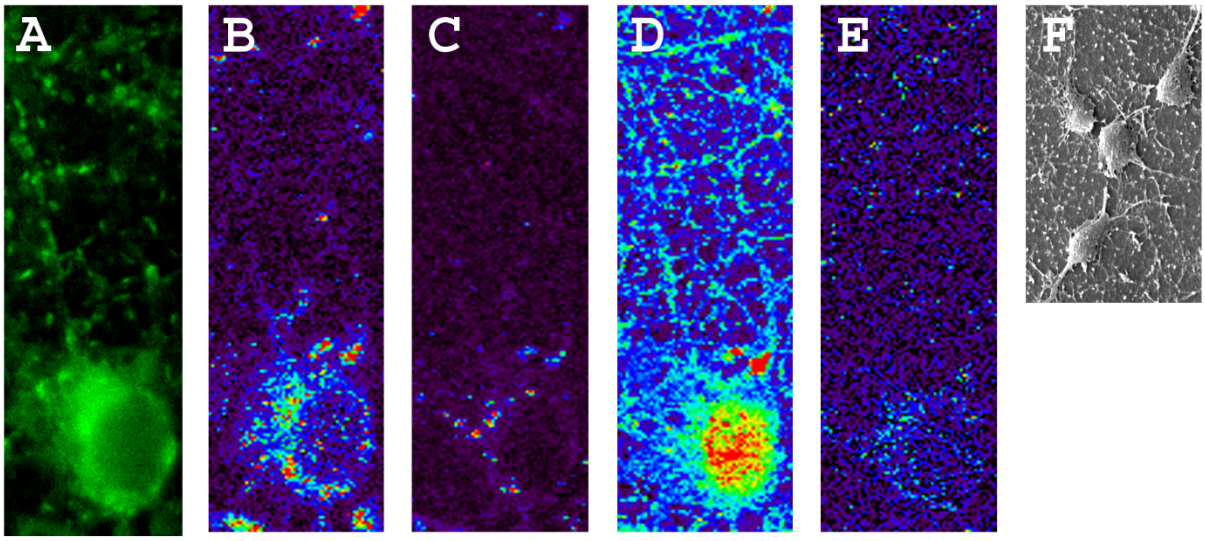
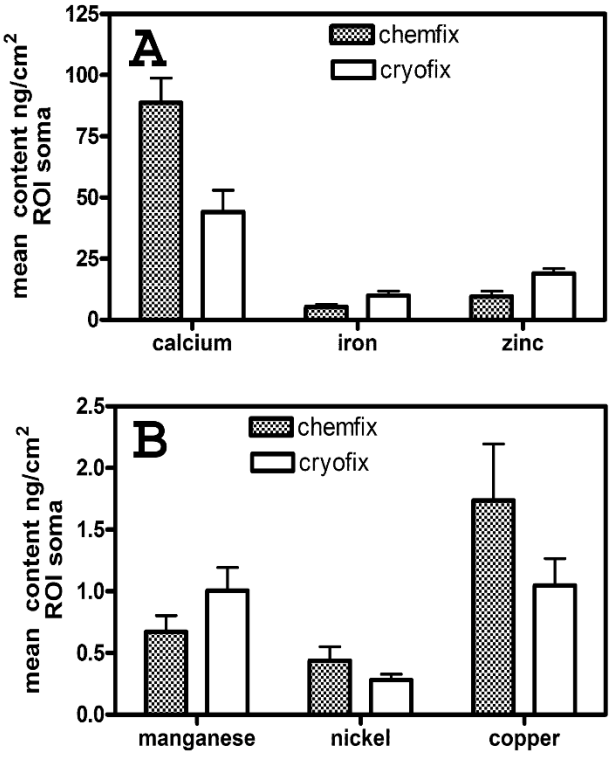


Figure S3: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