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Research Paper

On the nature of the Cu-rich aggregates in brain astrocytes

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ABSTRACT

Fulfilling a bevy of biological roles, copper is an essential metal for healthy brain function. Cu dyshomeostasis has been demonstrated to be involved in some neurological conditions including Menkes and Alzheimer's diseases. We have previously reported localized Cu-rich aggregates in astrocytes of the subventricular zone (SVZ) in rodent brains with Cu concentrations in the hundreds of millimolar. Metallothionein, a cysteine-rich protein critical to metal homeostasis and known to participate in a variety of neuroprotective and neuroregenerative processes, was proposed as a binding protein. Here, we present an analysis of metallothionein(1,2) knockout (MTKO) mice and age-matched controls using X-ray fluorescence microscopy. In large structures such as the corpus callosum, cortex, and striatum, there is no significant difference in Cu, Fe, or Zn concentrations in MTKO mice compared to age-matched controls. In the astrocyte-rich subventricular zone where Cu-rich aggregates reside, approximately 1/3 as many Cu-rich aggregates persist in MTKO mice resulting in a decrease in periventricular Cu concentration. Aggregates in both wild-type and MTKO mice show XANES spectra characteristic of Cu_xS_y multimetallic clusters and have similar [S]/[Cu] ratios. Consistent with assignment as a Cu_xS_y multimetallic cluster, the astrocyte-rich SVZ of both MTKO and wild-type mice exhibit autofluorescent bodies, though MTKO mice exhibit fewer. Furthermore, XRF imaging of Au-labeled lysosomes and ubiquitin demonstrates a lack of co-localization with Cu-rich aggregates suggesting they are not involved in a degradation pathway. Overall, these data suggest that Cu in aggregates is bound by either metallothionein-3 or a yet unknown protein similar to metallothionein.

1. Introduction

Owing to the brain's extreme metabolic demands and the wide range of biochemistry facilitated by copper (Cu), significant amounts of Cu are involved in the brain's function [13]. Serving as a cofactor of key proteins involved in mitochondrial activity, neurotransmitter and neuropeptide biosynthesis, oxidative stress defense and other critical brain processes, Cu is essential for normal brain function [50,64]. Redox active copper cycles between the cuprous (I) and cupric (II) forms to accomplish catalytic functions in proteins. Elevated Cu concentration has been noted in synaptic vesicles and its release during synaptic transmission has been suggested to play a role in synaptic plasticity and long-term potentiation [19,23]. Cu ions can also be damaging for the organism if they participate in reactions which generate oxidative stress. Since free Cu ions are toxic to the cell, sophisticated Cu transport and Cu chaperone systems have developed which involve proteins with extremely high Cu affinities [21]. Studies in yeast have found on average less than one free Cu ion per cell [55]. It is not surprising that perturbations to Cu homeostasis have been implicated in a number of devastating neurodegenerative diseases. Menkes and Wilson's diseases, for instance, have been shown to be manifestations of Cu deficiency and Cu overload caused by mutations in the copper transporting proteins ATP7A and ATP7B, respectively [12,72]. Additionally, there is an extensive body of literature implicating metal dyshomeostasis – in particular Cu and Zn - with the formation of plaques in the brain during neurodegeneration [22,3,40].

We demonstrated Cu-rich aggregates in glial fibrillary acid protein-positive (GFAP+) astrocytes in the subventricular zone [52,53] and other brain areas such as the hippocampus and rostral migratory steam of rats [66]. Further characterization of Cu rich aggregates showed that

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they are about a micron in diameter and can achieve localized concentrations of hundreds of millimolar [66]. The results on the Cu distribution in rodent brains were later verified by others using XRF [51] and LA-ICP-MS [20,37,6]. In the brain, astrocytes are thought to play a key role in copper homeostasis [57]. Possible pathways for Cu transport into the brain astrocytes include Cu absorption either from the blood or the cerebrospinal fluid (CSF). Astrocytes have perivascular end-feet or foot-plates, expanses of their cytoplasmic processes that surround the abluminal surfaces of the capillary endothelial cells that form the blood brain barrier (BBB) of the brain and are opposed to the endothelial basal lamina [1]. The BBB is partially lost on the surface of blood capillaries in the subventricular zone (SVZ) [67] which can explain the highest Cu accumulations detected in astrocytes positioned close to the ventricle wall [52,53]. Some of the astrocytes in the SVZ retain direct exposure to the surface of the ventricle wall even in adults [38]. Thus, astrocytes have preferential (as compared to neurons) access to the interstitial fluids as well as the CSF. As such, it has been proposed that astrocytes can serve as Cu "depots" for the brain [57,68] by accumulating metal which can later be used by the astrocyte or routed to neurons. Supporting this argument, it is important to note that astrocytes are capable of Cu uptake and export [58,59]. It is commonly believed that glutathione and metallothioneins – a family of metal-binding, cysteine rich proteins – are imperative to astrocytes' ability to buffer Cu [57].

To gain insight into the formation mechanism and biological significance of Cu-rich aggregates, it would be beneficial to determine their protein composition. XRF analysis of the [S]/[Cu] ratio and XANES spectroscopic analysis suggested Cu binding in the form of Cu_xS_y multimetallic cluster. Metallothionein (MT) was identified as a candidate binding protein [53]. Thus, we sought to characterize the metal distribution in a MT(1,2) knockout (MTKO) mouse in combination with other biological markers. Note that MT has three isoforms present in the central nervous system (CNS). MT1 and MT2 are regulated and produced coordinately, and are often described together as one functional entity. They are present primarily in GFAP+ glial cells in the brain [42,43,75] while MT3 is present in both astrocytes and neurons [28,74]. The MTKO mouse, which contains a disruption vector at the MT1 and MT2 genes, was originally reported to have increased sensitivity to transition metal toxicity, especially Cd, and poorer mitigation of oxidative stress [18,36,73]. Further studies on the MTKO phenotype in the brain have also implicated metallothionein in inducing reactive gliosis [34] and aiding in recovery from brain injury [17,48,70].

Using XRF, we report spatial quantification of biologically relevant metal ions in the brains of MTKO mice as compared to age-matched wild type (WT) mice. It is demonstrated that MTKO mice possess Cu-rich aggregates in the SVZ. The absence of MT(1,2), however, results in fewer aggregates in the SVZ though the remaining aggregates exhibit similar Cu concentrations in both MTKO and WT mice. Cu-rich aggregates in MTKO mice brains, however, exhibit similar [S]/[Cu] ratios and XANES spectra as WT mice, suggesting Cu is bound in similar Cu_xS_y multimetallic clusters. According to previous literature Cu_xS_y clusters formed with biomolecules display autofluorescence [45]. We indeed find that GFAP+ cells in the SVZ show autofluorescence following UV (350 nm) excitation. Autofluorescing bodies are similar in shape and size to the detected Cu-rich aggregates. Overall the data strongly suggest that Cu in Cu-rich aggregates is bound to metallothionein or a protein similar to metallothionein. Similarly, the data demonstrate that metallothionein plays a role in their formation with MT3 likely fulfilling the role of metallothionein(1,2) in MTKO mice.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats were purchased from Harlan (Indianapolis). Male 129S1/SvImJ (wild type) and 129S7/SvEvBrd-$MT1^{tm1Bri}MT2^{tm1Bri}$ mice (MTKO), an MT(1,2) double knockout mouse) were purchased from Jackson Labs [36]. Rats were sacrificed at 12 weeks old. Mice were sacrificed at 3 or 9 weeks old, as specified in the text. Immediately following extraction, the whole brain was snap frozen on liquid nitrogen and stored at −80 °C until sectioning. All experiments complied with animal rights regulations and were approved by the Institutional Committee on Animal Use at Purdue University.

2.2. Preparation of brain sections

Frozen sections were prepared in a Shandon FE/SE cryomicrotome (Thermo Scientific). To prevent sample contamination, Teflon coated blades were used. Samples were not fixed or chemically treated in any way. After being cut, sections containing the lateral ventricle (bregma=−0.8 mm) were placed on either glass microscope slides for conventional microscopy or X-ray compatible substrates made of 4 μm thick polypropylene film stretched flat on a frame for XRF microscopy. Sample thickness was either 30 μm (XRF, 8-BM, 18-ID), 10 μm (optical microscopy, XRF 2-ID-D/E and 20-ID). Samples were stored at −80 °C prior to use.

2.3. Synchrotron based X-ray fluorescence (XRF) microscopy

Elemental mapping was performed at the Advanced Photon Source (APS). A description of the BioCAT microprobe (beamline 18-ID) beamline is available at [4], a description of the x-ray microprobe (2-ID-D) at [15]. For imaging parameters used in the presented data, see Table 1. Focusing was done by KB mirrors (8-BM, 18-ID, and 20-ID) or Fresnel zone plates (2-ID-D/E) to the spot size specified in Table 1. The fluorescence spectra were recorded by an energy resolving silicon drift detector and fitted used the program MAPS [71]. Spectra are normalized by incident flux as measured by an ionization chamber. Quantitation was carried out using the NIST thin film standards NBS-1832 and NBS-1833 [46] or the AXO thin film XRF standard (AXO GmbH, Dresden).

Table 1
X-ray fluorescence microcopy imaging parameters.

Figure	Beamline	Pixel size μm^2 (v)×(h)	Beam size μm^2 (v)×(h)	Dwell time (sec)	Flux (photons/s)	X-ray energy (keV)
1A,Table 2(3 week)	**18-ID**	**20×20**	**5×5**	**0.05–0.20**	**2×10^{13}**	**10.0**
Table 2 (9 week)	**8-BM-B**	**25×25**	**25×25**	**0.25**	**4×10^{10}**	**10.0**
1B,Table 3	**18-ID**	**5×5**	**5×5**	**0.05–0.20**	**2×10^{13}**	**10.0**
1C,Table 4	**2-ID-D**	**0.3×0.3**	**0.2×0.25**	**1.0**	**4×10^{9}**	**10.0**
2, Inset	**20-ID-B**	**10×10**	**7×7**	**0.2**	**2×10^{12}**	**10.0**
2, Spectra	**20-ID-B**	**n/a**	**7×7**	**1–2**	**2×10^{12}**	**8.88–9.22**
4B	**2-ID-D**	**0.3×0.3**	**0.3×0.3**	**3.0**	**4×10^{8}**	**13.0**
4E	**2-ID-E**	**0.3×0.3**	**0.3×0.3**	**1.5**	**4×10^{8}**	**12.7**