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Characterization of Cu-rich aggregates in neurogenic niches of the rodent brain by X-ray fluorescence microscopy

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CHARACTERIZATION OF CU-RICH AGGREGATES IN NEUROGENIC NICHES OF THE RODENT BRAIN BY X-RAY
FLUORESCENCE MICROSCOPY

For the degree of Doctor of Philosophy

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Date

CHARACTERIZATION OF CU-RICH AGGREGATES IN NEUROGENIC
NICHES OF THE RODENT BRAIN BY X-RAY FLUORESCENCE
MICROSCOPY

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Brendan T. Sullivan

In Partial Fulfillment of the

Requirements for the Degree

of

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Live Mas.

-Taco Bell

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TABLE OF CONTENTS

	Page
LIST OF TABLES	viii
LIST OF FIGURES	ix
ABBREVIATIONS	xv
ABSTRACT	xviii
1 Copper and the mammalian brain	1
1.1 The roles of copper in the healthy brain	1
1.2 Copper transporting, trafficking, and storage in the brain	3
1.3 Copper and disease	6
1.4 Metallothioneins	8
1.5 Neurogenesis in the adult subventricular zone	9
2 Methodologies	11
2.1 Methods for mapping brain metal distributions	11
2.1.1 X-ray fluorescence microscopy (XRF)	12
2.1.2 Energy dispersive spectroscopy (EDS)	14
2.1.3 Electron energy loss Spectroscopy (EELS)	15
2.1.4 Laser ablation induced coupled plasma mass spectrometry (LA-ICP MS)	15
2.2 High-resolution X-ray diffraction mapping as an imaging modality	16
2.3 Synchrotron radiation for X-ray microscopy	19
2.3.1 X-ray production	20
2.3.2 Focusing: KB mirrors, compound refracting lenses, and zone plates	23
2.3.3 Silicon drift detectors	26
2.3.4 X-ray absorption near edge structure (XANES)	29
2.4 Immunohistochemistry: a (very) brief primer	30
2.5 Brain extraction and section preparation	32
2.6 Statistical analysis	34
3 Localization of Cu-rich aggregates in the rodent brain	37
3.1 Cu-rich inclusions are found in cells in neuroproliferative zones	38
3.1.1 Experimental details	39
3.1.2 The distribution of Cu aggregates in rats and mice in neuroproliferative zones	40

	Page
3.2 Cu-rich inclusions reside in glial-fibrillary acidic protein-positive (GFAP+) cells	46
3.2.1 Experimental details	47
3.2.2 Results of GFAP/Cu aggregate co-localization	48
3.3 Cu-rich inclusions are not found in lysosomes or ubiquitinated . . .	51
3.3.1 Experimental description	52
3.3.2 Results of Cu-autophagic marker co-localization	52
4 The composition of Cu-rich aggregates in the brain	55
4.1 Cu in aggregates is present as Cu(I)	55
4.1.1 Experimental description	56
4.1.2 Results of Cu-XANES in the rat SVZ	57
4.2 Nanoprobe XRF analysis of Cu inclusions	60
4.2.1 Experimental description	60
4.2.2 Results of nanoprobe analysis of Cu inclusions	61
4.3 Iron and Cu separately form the basis for pseudoperoxidase activity in the subventricular zone	65
4.3.1 Experimental Details	65
4.3.2 Results of DAB-XRF co-localization	66
4.4 X-ray diffraction of aggregates reveals that aggregates have periodic structure	68
4.4.1 Experimental description	69
4.4.2 Results of diffraction from Cu-rich aggregates	70
5 The response of Cu in the subventricular zone to aging and insults	73
5.1 Aging results in increased Cu in the subventricular zone	73
5.1.1 Experimental description	74
5.1.2 Results of aging XRF quantitation	75
5.2 Metallothionein(I,II) knockout mice exhibit fewer Cu aggregates in the SVZ	78
5.2.1 Experimental description	79
5.2.2 Results for metal quantitation of metallothionein knockout mice	80
5.3 Mn exposure does not alter Cu concentrations in the SVZ or neurogenesis	83
5.3.1 Experimental description	84
5.3.2 Results of Mn exposure on subventricular Cu and neurogenesis	86
5.4 Cuprizone does not alter Cu concentrations in the subventricular zone or corpus callosum	92
5.4.1 Experimental details	93
5.4.2 XRF and XRD quantification of cuprizone fed mice	93
6 Conclusions and future directions	97
REFERENCES	100

	Page
A Summary of XRF images used in figures	119
VITA	121

LIST OF TABLES

Table	Page
1.1 Copper binding proteins and assumed functions	2
2.1 Limits of current elemental imaging techniques	11
2.2 Edge and fluorescence energies of elements for brain imaging	14
2.3 Focusing method used at each beamline	27
2.4 List of primary antibodies presented in this work.	35
3.1 Cu Concentrations of Cu-rich inclusions in the SVZ	41
3.2 Copper concentrations in the SVZ and RMS	43
3.3 Species summary of Cu inclusions in Neuroproliferative Zones	46
4.1 S/Cu molar ratios at different imaging conditions	63
5.1 Cu quantitation in rats	77
5.2 Comparison of WT and MTKO mice	81
5.3 Mean Cu concentrations following Mn exposure ($\mu\text{g/g}$)	88
5.4 Quantitation in cuprizone mice	96
A.1 Details of XRF images	119

LIST OF FIGURES

Figure	Page
1.1 Diagram of the major proteins involved in Cu transport. Abbreviations are given in the abbreviations list in the front matter.	4
2.1 Left: Mechanism behind X-ray fluorescence. (1) A core electron is ejected by the incident X-ray and (2) filled by a higher lying electron. One such decay path is the release of a fluorescent photon. Right: Diagram showing allowed transitions and their name in Siegbahn notation. Energies are not drawn to scale.	13
2.2 Radial Integration to form diffraction maps. A,B,C. Representative diffraction images from different regions of a scan. The corpus callosum (A) has very high myelin concentrations, the anterior olfactory nucleus (B) which does not have an elevated myelin concentration, and the mylar substrate (C) which should be devoid of any myelin or neurofilaments. D-F. Radially integrated intensities for images A-C (blue dots) and their fit to a double Gaussian model (red line). G. The amplitude of the Gaussian at $d \approx 40$ Å (corresponding to myelin) plotted to form a myelin map with a pixel size of 100 μm . Inset letters correspond to diffraction images A-C. H. d spacing map of the same image.	18
2.3 Azimuthal integration for fiber orientation determination. A. Schematic representation of azimuthal integration. Each ring can be integrated as a function of ϕ around the ring and the resulting intensities will show any preferred orientations. B. Heat map of pixels to be considered for integration of two rings with the color representing the angle assigned to it. C. Example of fitting with a strong preferred orientation. Mean counts is shown in black dots with the fit of the Paris model in red. D. Example of fitting of a ring with no strongly preferred orientation. E,F. Myelin intensity maps from a rat brain (E) and the orientation map from the same scan (F) showing that this fitting scheme can reliably detect fibers preferred orientation while maintaining spatial information.	19
2.4 Schematic of a compound refracting lens (CRL).	24
2.5 Schematic of a zone plate. A. Drawing of a zone plate, which consists of alternating dark and transparent layers concentric rings. The farther rings are from the center, the narrower rings have to be to achieve focusing. B. Figure illustrating the path length different for the n th ring.	25

Figure	Page
2.6 A: Schematic of an antibody. B: Schematic for IHC. The primary antibody binds to the desire antigen, and the secondary antibody binds to the primary antibody. Since the secondary antibody is fluorescently labeled, protein expression can be probed using this technique	31
2.7 A. Sagittal section from a rat brain Lateral 1.9 mm. The lines indicate the sections shown in B and C. B. Coronal rat brain section at Bregma - 0.8 mm. C. Coronal rat brain section at Bregma - 2.56 mm. Abbreviations are given in the list of abbreviations.	34
3.1 Cu XRF maps from coronal rat brain sections at Bregma = -0.8 mm (A) and Bregma - 2.56 mm (B)	40
3.2 Cu inclusions in the SVZ. A. XRF microscopy in mouse brain clearly demonstrates intracellular structures with dramatically increased Cu content. The cell nucleus is identified by the increased phosphorous signal, while sulfur is distributed throughout the cytoplasm. Potassium has much higher concentration inside the cell than outside and, thus, helps to define the overall cell shape. B. XRF image of the SVZ. Elemental concentrations are in $\mu\text{g} / \text{cm}^2$ in 10 μm thick sections. The dashed line shows the ventricle wall with brain tissue in the right low corner. From XRF imaging it is evident that SVZ selectively accumulates Cu over other trace elements. C. XRF rat brain recorded with increased resolution along the ventricle wall showing that the Cu is also present in rats as intracellular aggregates. Cu K_{α} fluorescence is shown in red while Compton scattering is in cyan. The white dashed line indicates the ventricle wall. D Image of the same section as (C) after XRF analysis at room temperature stained with cresyl violet demonstrates tissue integrity after exposure to X-rays.	42
3.3 Cu XRF maps of A. rat sagittal section and B. mouse sagittal section with 25 μm resolution. While Cu concentrations are higher in the SVZ for mice, Cu aggregates do not exist along the RMS. C. Cu XRF map of a rat RMS with 1.5 μm spatial resolution, showing that Cu is present as aggregates in the RMS. D. Cu XRF map of a mouse RMS with 10 μm resolution showing that there are no Cu-rich aggregates along the mouse RMS. Display range is given in $\mu\text{g/g}$ in 30 μm sections.	44

Figure	Page
3.4	
Distribution of Cu aggregates in the hippocampus. A. Dark field micrograph of a DAB-stained mouse section shows no aggregates. B. X-ray fluorescence maps of a mouse hippocampus also shows no Cu accumulations, confirming that the Cu aggregates present in rats and in the SVZ of both species are not present. C. Dark field micrograph of a DAB-stained rat section reveals positively-stained aggregates in the dentate gyrus. D. Similarly, elemental maps show dense Cu-inclusions in dentate gyrus of the rat hippocampus. Scale bars: 100 μm . Display ranges are in mM through a 10 μm thick section.	45
3.5	
A. Co-localization of optical fluorescence of immunochemically stained GFAP (green) and X-ray fluorescence from Cu (red). The white dashed line indicates the ventricle wall. B. Subcellular co-localization of Cu with GFAP as visualized by immunostaining with gold nanoparticles as a secondary antibody. C. Left: Schematic of the cellular architecture of the SVZ. Progenitor cells (A-C) in the SVZ lie adjacent to the ependymal cell (E) layer lining the lateral ventricles. Right: XRF image of cells located in the SVZ of a Sprague-Dawley rat treated with BrdU - a marker of actively dividing cells. BrdU identifies type A cells - migrating neuroblasts and type C cells - transit amplifying cells. Co-localization of Br and phosphorus X-ray fluorescence signals shows actively dividing cells (dashed circles). Areas occupied by dividing cells have a diminished number of Cu-accumulations. The black arrows indicate the ventricle wall.	49
3.6	
Co-localization of Cu inclusions with lysosomes (LAMP1) and ubiquitin (UBQ). A. Optical micrograph of LAMP1 staining showing that LAMP1 stains small puncta that reside mostly in cells, including GFAP+ astrocytes. B. XRF co-localization of Cu (green), S (red), and Au-labeled LAMP1 (blue) showing that Cu does not reside in lysosomes in the SVZ. C. Optical micrograph of ubiquitin staining in the SVZ. Ubiquitin also forms small puncta that reside in cells. D. XRF co-localization of Cu (green), S (red), and Au-labeled ubiquitin (blue). These data show that Cu inclusions are not ubiquitinated.	53

Figure	Page
4.1	<p>A. Schematic diagram of coronal section located at approximately Bregma -0.8 mm. The red box indicates the approximate area of the XRF image. B. XRF image of copper distribution demonstrates that Cu enrichment is localized to a thin layer along the lateral ventricle wall. XANES measurements (C) were taken on Cu accumulations, indicated by arrows. Spectra obtained from different Cu-accumulations are the same within signal-to-noise. The red line is the average of all XANES measurements taken on this sample. D. Intracellular Cu K-edge XANES spectrum at low temperature on the Cu accumulations in a rodent brain (red) recorded at the same experimental conditions as the spectrum of Cu-MT prepared from rabbit apo-MT (blue). The high degree of similarity indicates that Cu is present in brain cells in the form of a Cu^+-thiolate multimetallic cluster and suggests that MT serves as a Cu binding protein.</p>
4.2	<p>Nanoprobe analysis of Cu inclusions. A. A representative Cu hotspot imaged with $30 \times 30 \text{ nm}^2$ pixel size. It is evident that Cu and S co-localize strongly and that accumulations are intracellular. Units are $\mu\text{g}/\text{cm}^2$ in a $5 \mu\text{m}$ thick section. B. S/Cu molar ratio only from pixels in the hotspot. Fitting the data to a linear fit yields a S/Cu ratio consistent with assignment of MT(1,2) and/or MT3 (see table 1.) C. A close-up on the Cu hotspot featured in A. The three lines correspond to the Cu intensity profiles drawn in D. Yellow dots identify the peak locations when fit to a Gaussian distribution. D. Cu intensity profiles corresponding to the green (top), red (middle) and magenta (bottom) lines in C. All three lines are well described by fits to two Gaussians, indicating that Cu hotspots are spheroid-like objects with diameters of around $1 \mu\text{m}$.</p>
4.3	<p>A. XRF map of Fe at 30 nm resolution from an unstained mouse section. In untreated sections, Cu and Fe aggregates tend not co-localize. Scale bar: 500 nm B. Demonstration of particle detection on the Fe map in A. Clusters with arrows, which have 5 or more pixels, are used for co-localization analysis. Scale bar: 500 nm. C. Co-localization statistics showing Pearsons correlation coefficient for Cu-rich clusters (left) and Fe-rich clusters (right). A boxplot showing the median, 25th percentile, 75th percentile and extremal values is given along with a scatter plot for individual clusters imaged with 30 nm or 40 nm resolution is shown. D. Co-localization of Cu, Fe, and P with 40 nm resolution demonstrating a case of Fe-Cu co-localization in the left cluster ($r = 0.49$). Display ranges are given in mM through a $5 \mu\text{m}$ thick section.</p>
	59
	62
	64

Figure	Page
4.4 DAB-XRF Co-localization. A. Low-magnification micrograph of the DAB-stained section. The arrow points to the rostral corner of the ventricle, which was imaged. Scale bar: 100 μm . B. Untransformed optical micrograph of the rostral corner. The dashed line indicates the ventricle wall and the box outlines the area imaged with X-ray microscopy. Scale bar: 10 μm . C. Co-localization of Cu, Fe, and optical images showing that DAB+ granules often, but not always, co-localize with metal aggregates. Note that the optical image has been transformed as described in Section 4.3.1. Scale bar: 5 μm	67
4.5 XRD from Cu aggregates. A. Section of a virtual powder stack for diffraction images recorded from Cu aggregates. The red arrows highlight the peaks observed at 3.14 $\text{\AA}$, which is composed of individual Bragg peaks. B. Integrated diffraction profiles of aggregates (blue) and control tissue (green). The difference (red) shows several peaks exist in aggregates that do not in normal tissue, the most prominent being at 3.14 $\text{\AA}$	70
5.1 A. 3D visualization of normalized Cu K_{α} fluorescence demonstrates an increased copper signal along the lateral ventricle (lv) wall (indicated by the white line) with increased age. Data were obtained from 30 μm thick coronal sections of the brains of 3 weeks, 3 months and 9 months-old rats. Similar distributions were observed in all 9 animals (3 in each age group) used in this study. Experimental details are shown in Appendix A. Insets show visualization of Ki67 proliferation marker. B. Circles (blue): Mean Cu concentration in the SVZ as a function of age. Solid line shows fit to monomolecular growth model ($R^2 = 0.944$). Squares (black): Mean Cu concentrations in the cortex of the brain. Triangles (red): Mean Cu concentrations of an entire half-brain coronal section. The solid line in the latter two cases shows a linear regression. It is apparent from this plot that of the regions surveyed Cu accumulation in the brain happens largely in the SVZ throughout aging. C. Cu concentrations in the SVZ Cu hotspots determined by median thresholding. The linearity may suggest that Cu accumulation above baseline levels is carried out by a mechanism which does not change its deposition rate with time ($R^2 = 0.56$). D. Correlation between the Cu concentrations in the SVZ Cu hotspots and proliferation rate ($r = -0.99$).	76

Figure	Page	
5.2	Comparison of MTWT and KO metal concentration and proliferation. A. Intensity map of normalized Cu K_{α} XRF with 6 μm resolution demonstrates the difference in copper concentration along the lateral ventricle wall in control mice (WT) as compared to MTKO mice. White dashed lines indicate the ventricle wall. B. Cu XRF allows counting of hotspots along the ventricle wall. WT rodents exhibit 38% more hotspots per micron. Display range is in ppm. C. Average metal concentration in various regions of the brain for MTKO (colored) and WT (white) mice. Structures that can readily be identified from XRF maps are the corpus callosum (CC), cortex (CTX), striatum (CPu), and the ventricle. D. Comparison of periventricular metal concentrations in MTKO (colored) mice to WT (white) mice. Images of the SVZ with 5 μm resolution show diminished Cu concentration in the SVZ as a result of the mutation.	82
5.3	XANES from tissue of MTWT and MTKO rodents. A. Cu K_{α} XRF map from a typical rodent with 10 μm resolution. The lateral side of the lateral ventricle (LV) is outlined with the dashed line. Arrows indicate spots where XANES scans were recorded. B. Summed XANES scans for MTWT (red) and MTKO (blue) rodents recorded directly from tissue.	83
5.4	A. Diagram of Lateral 1.90 mm with the approximate location of the displayed XRF scan indicated by the cyan box. B. A typical XRF spectrum from an elemental map with elemental K-edges labeled. When an element labels two peaks, these are its K_{α} and K_{β} edges which were both prominent. Peaks that overlap (e.g. K and Ca) are deconvoluted by spectral fitting via the MAPS program. C. Cu XRF image with different brain regions labeled. Example of SVZ and RMS selection prior to widening (D) and after widening by ± 3 pixels in the four cardinal directions (E). Note that widening overestimates the spatial dimensions of both the RMS and SVZ, thereby including neighboring structures (i.e. STR, CC, and/or LV). F. Results after applying a concentration threshold of 8.5 $\mu\text{g/g}$. Display ranges are in $\mu\text{g/g}$	87
5.5	A. Representative Ki67 stain of the SVZ and RMS for a control rat and B. Representative Ki67 stain for a Mn treated rat. C. High magnification (40 $\times$) view of the RMS in a control rodent. D. Quantitation of neurogenesis by Ki67+ cell density in the SVZ and RMS. No significant changes are observed with $n = 4$ animals in each group.	89
5.6	Combined XRF and XRD Analysis in the CC. A-C. Myelin map, Cu map, and ROI selection for the CC for a representative section. D. Quantitation of metals in the CC show no significant changes. E. Quantitation of mean myelin concentration in the CC; differences between groups are significant at $p < 0.05$	94

ABBREVIATIONS

A β	Amyloid beta
AAS	Atomic absorption spectroscopy
ADP	Adenosine diphosphate
ALS	Advanced Light Source
ANOVA	Analysis of variance
aNSC	Adult neural stem cell
APP	Amyloid precursor protein
APS	Advanced Photon Source
ATP	Adenosine triphosphate
Atox1	Antioxidant protein 1
BBB	Blood brain barrier
BCSB	Blood cerebrospinal fluid barrier
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
BSA	Bovine serum albumin
CC	Corpus callosum
CCO	Cytochrome <i>c</i> oxidase
CCS	Copper chaperone for superoxide dismutase
CN	Caudate Nucleus
C. Pal	Caudal palladium
CRL	Compound refracting lens
CTR1	Copper transporter 1
DAB	3,3'-Diaminobenzidine
DBH	Dopamine β -hydroxylase
DG	Dentate gyrus
DIC	Differential interference contrast

DMT1	Divalent metal transporter 1
EDS	Energy dispersive spectroscopy
EELS	Energy electron loss spectroscopy
ESC	Embryonic stem cell
FALS	Familial amyloid lateral sclerosis
Fzd	Frizzled
GFAP	Glial fibrillary acidic protein
GP	Gomori positive
GSH	Glutathione
HPC	Hippocampus
HPCf	Hippocampal formation
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IP	Intraperitoneal
JAK	Janus kinase
KB	Kirkpatrick-Baez (mirror)
LAMP	Lysosomal associated membrane protein
LA-ICP MS	Laser ablation-inductively coupled plasma mass spectrometry
LIF	Leukemia inhibitory factor
LV	Lateral ventricle
MS	Multiple sclerosis
MT	Metallothionein
MTKO	Metallotionein(1,2) knockout mouse
NSC	Neural stem cell
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
ppm	Parts per million
PrP	Prion Protein
RMS	Rostral migratory stream

SOD	Superoxide dismutase
SR	Synchrotron radiation
STAT	Signal Transducer activator of transcription
STR	Striatum
SVZ	Subventricular zone
TCF/LEF	T-cell factor/lymphoid enhancing factor
UBQ	Ubiquitin
UPS	Ubiquitin-proteosome system
WT	Wild type
XAFS	X-ray absorption fine structure
XANES	X-ray absorption near edge structure
XRD	X-ray diffraction
XRF	X-ray fluorescence

ABSTRACT

Sullivan, Brendan T. PhD, Purdue University, May 2016. Characterization of Cu-rich aggregates in neurogenic niches of the rodent brain by X-ray fluorescence microscopy. Major Professor: Yulia Pushkar.

Copper is an essential element in the brain playing several critical roles ranging from neurotransmitter synthesis to ATP production. As Cu is typically present in micromolar concentrations and has a spatially capricious distribution in the brain, determining concentrations has historically been challenging. X-ray fluorescence microscopy (XRF) offers excellent spatial resolution (down to 30 nm) and detection limits (sub parts per million), making it an excellent tool for analyzing metal distributions in the brain. Using XRF, it is demonstrated that Cu-rich aggregates with concentrations in the hundreds of millimolar are present in the subventricular zone of rats and mice. As the subventricular zone is a neurogenic niche in the adult brain, the hippocampal dentate gyrus and rostral migratory stream were also examined to see if these aggregates were present. It is shown that rats, but not mice, have Cu accumulations in the rostral migratory stream and dentate gyrus, which may be related to the fact that the rat hippocampus produces more neurons and new neurons are integrated more readily into neural circuits. In addition, these aggregates are found in glial fibrillary acidic protein-positive cells and a lack of co-localization with bromodeoxyuridine suggests they are specifically in type-B progenitor cells in the SVZ. Co-localization experiments with lysosomal associated membrane protein 1 and ubiquitin suggest that Cu aggregates are not simply awaiting autophagy but likely serve some purpose. (XANES) spectra recorded directly from Cu aggregates in tissue show that Cu is present as a Cu_xS_y multimetallic cluster and has the oxidation state Cu^+ . Consistent with being bound as a cuprous-thiolate cluster, Cu aggregates consistently co-localize with sulfur with a molar ratio of $[\text{S}]/[\text{Cu}] = 1.66 \pm 0.07$. By co-localizing

XRF maps and DAB staining, it is shown that Cu and Fe form exclusive aggregates in the subventricular zone that exhibit peroxidase or pseudoperoxidase activity.

Towards determining the role of Cu aggregates, the metal distribution in the subventricular zone was examined under several conditions and insults. The subventricular zone is shown to selectively accumulate Cu with age owing in part to the increase of Cu concentrations within aggregates. Metallothionein is a sulfur-rich protein known to be critical in Cu homeostasis in astrocytes cells. Metallothionein(1,2) knockout mice exhibit lower subventricular Cu concentrations owed in part to the fact that there are about one third as many aggregates in the SVZ and remaining aggregates contain less Cu. As another metallothionein isoform – metallothionein 3 – is present in the SVZ, it is likely that it fulfills the role of metallothionein(1,2). While assignment is metallothionein as the binding protein is consistent with literature, its exact role of the protein in aggregate formation remains unclear; metallothioneins may simply facilitate Cu uptake into the cell. Exposure to Mn, which is known to disrupt brain barriers, did not affect Cu concentrations for rats in either the subventricular zone or rostral migratory stream. Furthermore, the cuprizone mouse model, which reproduces demyelination as observed in multiple sclerosis through dietary Cu chelation, is examined. While X-ray diffraction mapping shows that myelin-rich areas show diminished myelin there is no corresponding decrease in Cu in these regions, suggesting that Cu starvation may not be the mechanism behind demyelination. Dietary Cu chelation by cuprizone did not affect metal concentrations in the subventricular zone.

Taken together, the results demonstrate that periventricular Cu accumulations are formed by a robust mechanism and likely serve some role. While the mechanism remains unclear, it is plausible that the aggregates somehow play a role in the neurogenic activity in the regions they are present.

1. Copper and the mammalian brain

The brain demonstrates an incredible capacity to accumulate Cu, exhibiting the highest concentrations of any tissue besides the liver [1]. Transition metals including Cu are critical to numerous processes in the brain ranging from neurotransmitter synthesis to antioxidant defense. Similarly, perturbations to metal homeostasis have been implicated in a wealth of diseases ranging from Alzheimer's disease to Menkes disease. As such, transition metals in the brain are tightly regulated both between brain regions and within subcellular compartments. The aim of this section is to provide an overview of the roles that Cu plays in the brain as well as the machinery regulating brain Cu homeostasis. Additionally, since much of the work presented in the thesis will be examined with respect to neurogenesis in the subventricular zone, a short introduction is given in Section 1.5.

1.1 The roles of copper in the healthy brain

Copper is responsible for many cellular and extracellular processes in the brain. An (incomplete) list of non-transporting Cu-binding proteins present in the brain is presented in Table 1.1. A common role for Cu in cuproproteins is to act as an oxidoreductase. As an important example, consider superoxide dismutases 1 and 3 (SOD) which can neutralize superoxide (O_2^-) by the following half-reactions:

Superoxide is regularly generated as a byproduct of several brain functions (e.g. by mitochondrial respiration [2]) which will ultimately lead to cell death if not rendered into a more stable form [3]. As a result, the importance of SOD and proper Cu

homeostasis cannot be overstated. Mutations to Cu,Zn SOD, for instance, have been strongly implicated in familial amyotrophic lateral sclerosis (FALS) [4].

Table 1.1.
Copper binding proteins and assumed functions

Protein	Assumed Function	References
Cytochrome <i>c</i> oxidase	ATP Production	[5, 6]
Metallothionein	See Section 1.4	[7–9]
Dopamine β -hydroxylase	Neurotransmitter synthesis	[10, 11]
Ceruloplasmin	Fe metabolism	[12]
Cu,Zn Superoxide Dismutase	Antioxidant defense	[13, 14]
Lysyl Oxidase	Collagen and Elastin Production	[15, 16]
Prion	Unknown	[17–19]
Amyloid Precursor Protein	Unknown	[20–22]
α -Synuclein	Unknown	[23–25]

Another critical example of Cu's role as an oxidoreductase in the brain is during ATP production. Cellular respiration in the brain occurs primarily in mitochondria, with estimates that more than 90% of the brain's energy is generated by mitochondria [26, 27]. The mitochondrial pathway to ATP production is driven by an electron transport chain that energetically enables ATP production by ATP synthase:

The final step in the electron transport chain is carried out by the transmembrane enzyme cytochrome *c* oxidase which contains 2 Cu sites [5] serving as electron acceptors from cytochrome *c* and enabling oxidation of the previously reduced cytochrome *c* [28]. This reaction is critical in maintaining cellular metabolism in all forms of life. The

importance in the brain is emphasized in situations when Cu availability prevents proper cytochrome *c* oxidase activity (Section 1.3).

The role of Cu as an oxidoreductase also renders Cu homeostasis important to cell signaling. The dependence of Cu availability on cell signaling arises by several mechanisms. First, Cu can be present at the active site of enzymes related to signaling. As an example, dopamine β -hydroxylase (DBH), responsible for converting dopamine to norepinephrine, has two Cu ions at its active site and functions as an oxidase in the reaction [10,11]. Secondly, exogenous Cu^{2+} has been shown to act as an antagonist to a variety of signalling receptors including γ -aminobutyric acid (GABA) [29, 30] and N-methyl-D-aspartate (NMDA) [31]. Finally, Cu can affect signaling by managing cellular oxidative stress which, in turn, affects transporter and receptor expression.

1.2 Copper transporting, trafficking, and storage in the brain

Having examined a handful of critical physiological roles of Cu in the brain, it is evident that healthy brain function relies heavily on Cu being delivered to the correct proteins. As unbound Cu ions would pose a serious threat to cells by inciting an avalanche of oxidative species – indeed the average yeast cell was found to have less than one free Cu ion per cell [32] – Cu must be carefully escorted to the correct cells and proteins. This is done by transmembrane Cu transporting proteins and cytosolic Cu chaperone proteins. First, Cu must be uptaken into the brain from either the blood-brain barrier (BBB) or blood-cerebrospinal fluid barrier (BCFB), both of which have been shown to transport Cu primarily as a free ion [33, 34]. A pictorial representation of the process is given in Figure 1.1.

Copper uptake into the cell has been shown to be primarily done by copper transporter 1 (CTR1) [35, 36] in *ex vivo* experiments, which is emphasized by the fact that CTR1 knockout is embryonically lethal [37]. DMT1, however, is capable of transporting Cu across the cell membrane [38]. Upon entry to the cell, Cu ions are sequestered by chaperones for transport to their protein destination or are sequestered by glu-

Figure 1.1. Diagram of the major proteins involved in Cu transport. Abbreviations are given in the abbreviations list in the front matter.

tathione (GSH). While only a handful of copper chaperones have been identified, they all share similar structural motifs and are conserved across different kingdoms highlighting their importance [39, 40]. The major chaperones are shown in Figure 1.1. Studies of chaperone knockout mice have demonstrated the importance of these proteins. Knockout mice for copper chaperone for superoxide dismutase (CCS), for instance, show no growth defects through adulthood, but female mice exhibited di-

minished fertility [41]; it is possible that other antioxidant defenses or glutathione (GSH) can compensate for the lack of activated Cu/Zn SOD. Other transporters are more critical. Antioxidant protein 1 (ATOX1) knockout mice, for instance, exhibit severe growth defects and seizures [42] while cytochrome *c* oxidase copper chaperone (COX17) knockout is embryonically lethal due to decreased mitochondrial respiration [43].

Within the cell, it is necessary to transport Cu ions across membranes either for cellular export or delivery to the *trans*-Golgi network. This is achieved primarily by ATP7A and ATP7B. ATP7A and ATP7B are transmembrane proteins that rely on ATP hydrolysis to actively pump Cu ions across the membrane [44]. ATP7A and ATP7B are critical to preventing dangerous cellular concentrations of Cu and ensuring that the ion can be incorporated into proteins. Loss-of-function mutations to ATP7A and ATP7B manifest themselves as Menkes and Wilson’s diseases, respectively, which are discussed in more detail in Section 1.3.

While the *in vivo* onus of Cu transport across brain barriers is not unambiguously defined, [45], there is strong evidence that astrocytes are responsible for uptake across the brain barriers. Astrocytes heavily regulate the properties of the blood brain barrier [46] and have the ability to uptake metals through their perivascular endfeet [47] via CTR1 and possibly DMT1 [33]. Similarly, it has been demonstrated that type-B progenitors, a subset of periventricular astrocytes (see Section 1.5), retain contact with the blood cerebrospinal fluid barrier [48], allowing preferential access to Cu at the barrier. As such, it has been proposed that astrocytes can serve as Cu “depots” for the brain [49,50] by accumulating metal which can be later used by the astrocyte or routed to neurons. Supporting this argument, it is important to note that astrocytes, at least in culture, are capable of Cu uptake [51] and export, likely via an ATP7A-dependent pathway [52].

In addition to their fortuitous placement along brain barriers, astrocytes exhibit impressive Cu storage abilities. The storage capacity is achieved through a combination of GSH and metallothioneins. GSH is a tripeptide of glutamine, cysteine,

and glycine that has been implicated in antioxidant activity, redox signaling, metal sequestration, and cell cycle regulation [53] that is preferentially present in astrocytes [54]. The malleable structure of GSH renders it capable of binding transition metals, including Cu. Additionally, it has been shown that Cu-GSH can act as a chaperone for several proteins. Metallothioneins (MTs) are a class of cysteine-rich proteins that bind up to twelve metal ions (see Section 1.4). Of particular interest for Cu storage is the fact that GSH has been shown to deliver Cu to MT [55]. It is widely believed that astrocytes' ability to effectively store Cu lies in quick sequestration by GSH with long term storage being fulfilled by MT, enabling timely delivery of Cu as needed. Copper concentrations have also been shown to regulate the expression of MT and GSH, making them excellent storage solutions as they can be produced on an as-needed basis.

1.3 Copper and disease

Given the important roles Cu fulfills and the the degree to which its transport is regulated, it is unsurprising that dysfunction in Cu handling machinery leads to disease. Menkes disease, for instance, occurs as a result of ATP7A deficit [56]. These disease, first published by Menkes in 1962 [57], affects patients through developmental retardation, muscle weakness, seizures, and wiry, brittle hair; patients rarely live last three years of age. Without adequate functioning ATP7A, Cu cannot be correctly uptaken from food in the small intestine nor can it be delivered to cuproenzymes - in particular cytochrome *c* oxidase deficiency has been proposed as a major source of Menkes symptoms [58]. The disease is currently untreatable, though subcutaneous Cu delivery has had limited clinical success in mitigating the effects. Intraventricular injections of viruses harboring ATP7A have been shown to aid significantly in extending the life of mottled brindled mice (a Menkes model) and increasing cuproenzymatic activity, suggesting that gene therapy may be a viable pathway for human treatment [59].

Wilson’s disease, on the other hand, is a Cu overload condition. First described in a monograph by Wilson in 1912 [60], the Cu overload was shown to be caused by mutations to ATP7B [61]. After dietary uptake, Cu is transported to the liver. In the liver, ATP7B is responsible for 1) packing Cu into ceruloplasmin for release into blood and 2) biliary excretion of excess Cu; deficient ATP7B then results in excess Cu accumulating in organs, including the brain [62]. Symptoms include cirrhosis of the liver, depression, neuroses, and Parkinson’s-like symptoms. Wilson’s disease is detectable by liver biopsy and by measuring ceruloplasmin and copper concentrations in blood and urine. Copper chelation therapy has proven successful in mitigating the effects of Wilson’s disease and, in extreme cases, liver transplants can recover Cu homeostasis.

Cu is also implicated in a number of aggregate-based diseases. As an example, Alzheimer’s disease – caused by the formation of insoluble amyloid plaques – are known to nucleate around Cu and Zn centers and indeed retain the metals in high concentration [22, 63, 64]. There is a growing body of evidence that Cu’s binding with α -synuclein accelerates the aggregation process [65], promoting the formation of Lewy bodies and ultimately Parkinson’s disease. Cu homeostasis is also thought to play a role in prion diseases – caused by the misfolding of the prion protein (PrP) into its pathogenic, scrapie form (PrP^{Sc}) – though the etiology is not yet clear [66]. Copper is known to compete with Mn for binding in PrP and the latter is known to induce oligimerization [67]. One possibility, then, is that Cu occupies sites that would otherwise be occupied by Mn and prevents rapid polymerization [68]. In each of these cases, the relationship between Cu and aggregate formation remains somewhat unclear, but metal binding may induce conformational changes that promote oligimerization which leads to aggregation.

1.4 Metallothioneins

Central to Cu homeostasis in the brain is metallothionein (MT). Metallothioneins are a class of cysteine-rich proteins that have been implicated in numerous brain functions. While MT was originally reported from equine kidney in 1957 [69] it was eventually determined to be ubiquitous amongst mammals and even present in some plants and prokaryotes [7]. There are four distinct isoforms of metallothionein, named MT1, MT2, MT3, and MT4. In mammals, MT1 and MT2 are expressed ubiquitously through the body while MT3 is expressed primarily in the central nervous system and MT4 is not expressed in the nervous system [70]; MT1 and MT2 are expressed preferentially in the astrocytes while MT3 is expressed primarily in neurons, though all three isoforms have been detected in each cell type [71].

Metallothionein's ability to bind up to twelve metal ions has been attributed to two distinct metal binding domains, one β strand and one α helix which bind metals using highly conserved cys-cys, cys-x-cys, and cys-x-y-cys residues [72]. These motifs have been attributed to metallothionein's high metal binding affinities; Cu^+ has been shown to have the highest affinity (10^{17} – 10^{19}) followed by Cd^{2+} (10^{15} – 10^{17}) and Zn^{2+} (10^{11} – 10^{14}) [73].

While the importance of MT to metal homeostasis, and therefore oxidative stress, has been recognized, a primary function aside from metal sequestration is yet to be determined. Within the brain, metallothioneins have been implicated in several processes. MT(1,2) knockout (MTKO) mice, for instance, are unable to quickly recover from cortical injuries or induced seizures, implicating MT in activating astrocytes and forming a glial scar around affected areas [74–76]. The neuroprotective role of metallothionein may also be a result of being exported from astrocytes and interacting with neurons [77, 78]. MT is also thought to be critical in managing apoptosis. One realization of this is by preventing release of cytochrome *c* from mitochondria, thereby preventing the caspase cascade route to cell death [79].

1.5 Neurogenesis in the adult subventricular zone

It was a long-held belief that the developed brain was a static structure; that is to say that after development no neurons could be produced. That belief was overthrown with Altman's 1962 seminal paper in which radioactively labeled thymidine was used to mark cells that had undergone division in adulthood [80]. Further studies showed that adult neurogenesis is a common trait in the mammalian brain [81] including humans [82]. Adult neurogenesis has been demonstrated in the subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampal formation (HPCf). In the subventricular zone, a subset of glial fibrillary acidic protein (GFAP+) astrocytes are the neural stem cells [83] with offspring capable of eventually becoming neurons, astrocytes, or oligodendrocytes [84]. These cells then migrate along the rostral migratory stream (RMS, see Figure 2.7) to the olfactory bulb, differentiating along the way. There are four major cell types involved in the process of proliferation, migration, and differentiation [83]:

- **Type A migrating neuroblasts** are fast moving neural precursor cells derived from type C cells. Neuroblasts are lineage committed to become a neuron, but are still capable of division unlike neurons, which are postmitotic.
- **Type B slow-progenitor cells** are the GFAP+ progenitor cells¹ in the subventricular zone. These cells define the migratory pathway to the olfactory bulb and a subset of these cells have been shown to be neural stem cells [85].
- **Type C transit amplifying cells** are derived directly from type B progenitors and will ultimately give rise to neuroblasts. Type C cells retain multipotency that can be activated in the presence of growth factors [86].
- **Type E ependymal cells** make direct contact with the ventricle and have cilia which serve to absorb and circulate cerebrospinal fluid. The choroid plexus is

¹A lack of *in vivo* markers for true neural stem cells has given rise to the term “progenitor cell”, which is used to describe cells with some differentiation potential, though these cells may not strictly be true stem cells.

composed primarily of modified ependymal cells. Ependymal cells have also been shown to be capable of neuron production, but are eventually depleted.

Following differentiation into neurons, survival and functional integration into neuronal circuits are also issues. While these are beyond the scope of the work here, reviews on the subjects are available.

There are a wealth of factors influencing cell proliferation in mice. Empirically, it has been demonstrated that exercise, stress, and sleep can all affect neurogenic rates, though the biochemical mechanism is not clear [87]. Exogenous application of many chemical agents including growth factors, cytokines, and neurotransmitters have all been reported to modulate neurogenic rates (which are nicely reviewed in [88].) The exact mechanisms by which those affect cell proliferation in neuroproliferative zones remains elusive. In mice models, primarily from *ex vivo* studies, it has been shown that a central component in maintaining cellular potency is the canonical Wnt signalling pathway. The pathway is reviewed extensively in [89]. Briefly, the canonical pathway involves Wnt, a signalling protein, binding to a frizzled (fzd) transmembrane receptor. The Wnt-fzd pair results in cytosolic accumulation and a subsequent translocation of β -catenin to the nucleus. In the nucleus, β -catenin binds to T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors. In murine stem cells, activation of the canonical Wnt pathway has been shown to upregulate STAT3 and prevent progenitor differentiation, instead promoting self-renewal [90,91]. It should be noted that in humans, STAT3 is not sufficient for self renewal [92]; Wnt is still thought to be important in human stem cell differentiation through mechanisms still under investigation.

2. Methodologies

2.1 Methods for mapping brain metal distributions

Having established the importance of mapping metal distributions in the brain, it is worthwhile to consider the challenges involved in measuring said distribution. In practice, there are three technical hurdles that must be overcome in order to reliably map metals in the brain. First, it is imperative that elements can be distinguished from other elements (e.g. differentiating Cu and Zn). Secondly, as concentrations are often in the micromolar range, the ideal method would have very high sensitivity. Finally, as discussed in Chapter 1, the brain exhibits a very capricious metal distribution and so high spatial sensitivity is required while still allowing measurements to be made over entire brain regions. Table 2.1 has an overview of pertinent parameters for elemental imaging in biological systems. Those more pertinent to brain imaging are discussed below. For a more complete discussion, the reader is referred to [93].

Table 2.1.
Limits of current elemental imaging techniques

Technique	Detection Limit ($\mu\text{g/g}$)	Spatial Resolution (μm)	Penetration Depth (μm)
XRF	0.1-1	0.03+	>100
PIXE	1-10	0.2-2	10-100
EDS/EDX	100-1000	0.03	0.1-1
EELS	1000	0.001	<0.05
LA-ICP MS	0.01	5+	200
SIMS	0.1	0.05	0.1
Radioisotopes	n/a	50-100	>1000

2.1.1 X-ray fluorescence microscopy (XRF)

X-ray fluorescence occurs when a non-valence electron is removed from the atom (in this case by X-ray bombardment) creating a core hole. This core hole is then filled by a higher energy electron and, in doing so, emits a photon with an energy equal to the energy difference between those two states (Figure 2.1). A diagram of transitions through the N shell is given in Figure 2.1. Transitions obey quantum selection rules. For the dominant electric dipole (E1) transition, these are given by

$$\begin{aligned}\Delta n &\geq 1 \\ \Delta l &= \pm 1 \\ \Delta j &= \pm 1\end{aligned}\tag{2.1}$$

Furthermore, cross section calculations have shown K-shell fluorescence to dominate L and M-shell fluorescence [94]. Consequently, XRF measurements are dominated by $p \rightarrow s$ transitions - i.e., K_α fluorescence. Other transitions are observed but have relatively low yields.

There are several requirements for a beamline to conduct a productive X-ray fluorescence microscopy experiment for trace elements in biological systems. First, X-rays must be produced with an energy above the absorption edges of interest and with adequate flux to allow timely measurements. For biological trace metal investigations, interest primarily lies in Cu, Fe, Mn, and Zn, whose K edges are given in Table 2.2. For the metals pertinent to this project (Table 2.2), an incident energy of 10 keV is adequate; conveniently, many microbeam imaging beamlines obtain high flux at 10 keV. If gold immunostaining (see Section 2.4) is being employed, Au is excited at the Au L edge (d to p orbital fluorescence), at 11.92 keV; these experiments are typically carried out with an incident energy of 13 keV for results shown in this work. Secondly, the incident beam must be focused to an appropriate size. Focusing, described in Section 2.3.2, is achieved using either a zone plate, a compound refracting lens (CRL) or Kirkpatrick-Baez (KB) mirrors. In practice, each beamline has optics tailored to a specific spot size and so the required resolution for an experiment de-

Figure 2.1. Left: Mechanism behind X-ray fluorescence. (1) A core electron is ejected by the incident X-ray and (2) filled by a higher lying electron. One such decay path is the release of a fluorescent photon. Right: Diagram showing allowed transitions and their name in Siegbahn notation. Energies are not drawn to scale.

termines at which beamline an experiment should be carried out. Lastly, a means to record the fluorescence with energy resolution that can resolve individual elements is necessary. In the work presented here, this is done with an energy dispersive silicon drift detector (Section 2.3.3). To make XRF measurements quantitative the recorded spectra must be compared with a known standard. In principle, any thin film containing the metal of interest at a known concentration is sufficient for normalization. In all data from the APS reported in this paper, either the (now obsolete) NBS1832 and NBS1833 thin film standards or AXO Thin Film XRF references are used for absolute concentration calibration. Information on the standards is available in [95] and from Axo-Dresden GmbH (Dresden, Germany), respectively.

Table 2.2.
Edge and fluorescence energies of elements for brain imaging

Element	K edge	K_{α} Fluores- cence	K_{β} Fluores- cence
P	2.15 keV	2.02 keV	2.14 keV
S	2.47 keV	2.31 keV	2.46 keV
K	3.61 keV	3.31 keV	3.59 keV
Ca	4.03 keV	3.69 keV	4.01 keV
Mn	6.53 keV	5.90 keV	6.50 keV
Fe	7.11 keV	6.40 keV	7.06 keV
Cu	8.98 keV	8.05 keV	8.94 keV
Zn	9.66 keV	8.64 keV	9.57 keV

2.1.2 Energy dispersive spectroscopy (EDS)

Energy dispersive spectroscopy (EDS) operates on the same principle as XRF - a fluorescence photon is emitted and recorded by an energy-sensitive detector. The only difference is that a focused beam of electrons are used as an excitation source rather than X-rays. The principle advantage of EDS is that it is routinely performed using electron microscopes, which are commercially available and access is relatively common. As such, very high spatial resolution (~ 30 nm) is readily achievable and access to equipment is not a concern.

The downside, however, lies in the detection limits (Table 2.1). Biological metal concentrations often fall in the μM range, below what can be reasonably detected by EDS. Furthermore, the low penetration depth of electrons in tissue sections (< 1 μm) does not allow sampling through entire cells in tissue sections. The issue of penetration depth can be circumvented by analyzing serial sections with thicknesses on the order of the penetration depth, which is commonly done in studying the ultra-

structure of the brain [96–98]. However, these approaches typically require fixation for sectioning and/or osmium staining for visualization, and as such will redistribute metal in the brain. As a result, XRF is preferred for imaging dilute biological systems such as brain sections.

2.1.3 Electron energy loss Spectroscopy (EELS)

Electron energy loss spectroscopy (EELS) is an imaging technique that determines elemental composition by measuring the energy lost when electrons inelastically scatter off the sample. In principle, EELS can provide information down to the atomic level [99]. However, the limited penetration depth (50 nm) and high detection limit renders it of limited use in tissue imaging. Even for single cell imaging, special treatment for ultra-thin section preparation and osmium staining for contrast can alter physiological metal concentration and localization.

2.1.4 Laser ablation induced coupled plasma mass spectrometry (LA-ICP MS)

Laser ablation induced coupled plasma spectrometry (LA-ICP MS) is an analytical technique in which a tightly focused, high power laser beam ablates the sample. The resulting particles are then ionized, typically by a plasma torch, and analyzed for elemental composition via mass spectrometry. By scanning the sample and measuring over a grid of points, elemental maps can be formed. It is noted from Table 2.1 that LA-ICP MS has superior detection limits and comparable penetration depth to XRF. The current limitation for LA-ICP-MS is spatial resolution. Currently achievable resolutions are a matter of technological limitations; the only fundamental limitation is the laser spot size which can be focused to roughly the wavelength of ablating light (often around 200 nm.) It should be noted, however, that there is a tradeoff between spot size and detection limits [100]. Owing to its wide field of view, low detection limits, and the fact that chemical treatment is not necessary for sample preparation,

LA-ICP MS has been used for imaging a wide range of biological systems [101–103], including the brain tissue [100, 104, 105].

2.2 High-resolution X-ray diffraction mapping as an imaging modality

Complementary to XRF measurements are X-ray diffraction mapping experiments. By recording a diffraction image at each point on a grid covering a brain section, it is possible to record a wealth of structural information directly from brain sections. One particular application of diffraction mapping in the brain is to analyze the fiber patterns produced by myelin and neurofilaments. Myelin, the insulating material surrounding neuronal axons, has a lamellar structure with a repeat of quasi-periodicity of 173 Å [106] while neurofilaments are intermediate filament proteins comprising the cytoskeleton of neurons that assemble into tetramers that repeat every 220 Å [107]. The constitution of these structures are critical to healthy neuronal signaling in the brain and their dysfunctions have been associated with a variety of diseases ranging from multiple sclerosis to amyloid lateral sclerosis.

Fiber diffraction patterns for both of those structures can be observed by recording diffraction in the 10 Å – 120 Å range, as done in this work. It is common to observe two rings from brain sections over that range of d – one at ≈ 43 Å and one at ≈ 58 Å. The former is associated with the fourth order reflection of myelin while the latter is a convolution of myelin’s third order reflection and the fourth order neurofilament reflection. The resulting diffraction image for each pixel is analyzed in two ways. First, it can be integrated radially. This results in a curve containing a peak for each observed diffraction ring in addition to some background. By assuming a functional form for both the background and the peaks, this curve can be fit; for simplicity the present work assumed a linear background and that peaks are shaped as Gaussians:

$$A_1 e^{\frac{(d-\tilde{d}_1)^2}{2\sigma_1^2}} + A_2 e^{\frac{(d-\tilde{d}_2)^2}{2\sigma_2^2}} + Bd + C \quad (2.2)$$

Once the profiles are fit (Figure 2.2D-F), the parameters can be used to create spatial maps (Figure 2.2G). The amplitude A_i , for instance, reports on the concentration of the species being fit (e.g. myelin).

Additionally, each ring may be azimuthally integrated to reconstruct the spatial orientation of each fiber (Figure 2.3). A geometric description of the azimuthal profile was described in 2003 by Paris and Müller [108]. In brief, the model gives a description of the intensity I as a function of ϕ , the polar coordinate on the detector face:

$$\begin{aligned} I(\phi) &= I(\Psi(\phi)) \\ \Psi(\phi) &= \cos^{-1}(\cos \theta \sin \mu \cos \phi - \sin \theta \cos \mu) \end{aligned} \quad (2.3)$$

With μ is the angle of the fiber axis with respect to the beam and the diffraction ring is observed at a diffraction angle 2θ . Then, assuming a Gaussian distribution of fiber orientations whose diffraction maxima form at ϕ_0 and have a standard deviation σ_ϕ and a background B , each ring profile can be fit as:

$$I(\phi) = A_\phi \exp\left(-\frac{\frac{\pi}{2} - (\cos^{-1}(\cos \theta \sin \mu \cos(\phi - \phi_0) - \sin \theta \cos \mu))^2}{2\sigma_\phi^2}\right) + B_\phi \quad (2.4)$$

To construct azimuthal profiles, rings are binned into angular bins (Figure 2.3B) and the mean counts of pixels in that bin is taken. Fits are carried out as non-linear least squares fits to data and fit quality is monitored by the sum of squared residuals. Radially and azimuthal fits were done separately with the radial fit being done first as the azimuthal fit depends on the resulting θ . Without peaks (e.g. Figure 2.3D), these profiles are described equally well by a large background B_ϕ with low amplitude A_ϕ or by very low background with a very broad distribution (large σ_ϕ). Physically these reduce to the same situation: a randomly oriented set of fibers in the sampled voxel. To remove this mathematical ambiguity, the bounds on azimuthal background are placed just below the mean intensity forcing a solution with high background. A representative orientation map of myelin around the ventricle is shown in Figure 2.3E,F.

Figure 2.2. Radial Integration to form diffraction maps. **A,B,C.** Representative diffraction images from different regions of a scan. The corpus callosum (A) has very high myelin concentrations, the anterior olfactory nucleus (B) which does not have an elevated myelin concentration, and the mylar substrate (C) which should be devoid of any myelin or neurofilaments. **D-F.** Radially integrated intensities for images A-C (blue dots) and their fit to a double Gaussian model (red line). **G.** The amplitude of the Gaussian at $d \approx 40$ Å (corresponding to myelin) plotted to form a myelin map with a pixel size of $100 \mu\text{m}$. Inset letters correspond to diffraction images A-C. **H.** d spacing map of the same image.

Figure 2.3. Azimuthal integration for fiber orientation determination. **A.** Schematic representation of azimuthal integration. Each ring can be integrated as a function of ϕ around the ring and the resulting intensities will show any preferred orientations. **B.** Heat map of pixels to be considered for integration of two rings with the color representing the angle assigned to it. **C.** Example of fitting with a strong preferred orientation. Mean counts is shown in black dots with the fit of the Paris model in red. **D.** Example of fitting of a ring with no strongly preferred orientation. **E,F.** Myelin intensity maps from a rat brain (E) and the orientation map from the same scan (F) showing that this fitting scheme can reliably detect fibers preferred orientation while maintaining spatial information.

2.3 Synchrotron radiation for X-ray microscopy

A typical X-ray microscopy experiment requires 1) an X-ray source, 2) a monochromator, 3) a focusing mechanism, and 4) a detector. Owing to the dilute nature of metals in biological systems, table-top X-ray sources make experiments prohibitively time consuming and so only synchrotron experiments will be considered here. For a more complete description, the reader is referred to [109].

2.3.1 X-ray production

Synchrotron radiation refers to X-rays generated by accelerating electrons traveling at relativistic speeds. Electron bunch acceleration is typically achieved by smaller accelerators prior to injection into the main storage ring. At the Advanced Photon Source, for instance, a linear accelerator and booster ring accelerate bunches to 7 GeV ($\gamma \approx 13,700$) prior to storage ring injection.

Once in the main storage ring, electrons are accelerated transverse to their direction of propagation to produce X-rays. X-ray production done by either an insertion device (ID) or bending magnet (BM), the former being designed explicitly for X-ray production and the latter for steering of electrons around the storage ring with X-ray production as fortunate consequence. Insertion devices are a series of magnets arranged to induce transverse oscillations in the electrons' trajectories. For an electron moving in the $\hat{z}$ direction being perturbed in the $\hat{x}$ direction due to a periodic magnetic field in the $\hat{y}$ direction, an electron's motion can be described by a Lorentz force:

$$\frac{d\mathbf{p}}{dt} = -e(\mathbf{E} + \mathbf{v} \times \mathbf{B}) \quad (2.5)$$

A series of alternating magnets are used to perturb the motion, so the assumption $E \ll B$ is reasonable:

$$\frac{d\mathbf{p}}{dt} \approx -e\mathbf{v} \times \mathbf{B} \quad (2.6)$$

Furthermore, electrons are moving at very near the speed of light along $\hat{z}$, such that $\mathbf{v} \approx v_z$. Assuming a periodic magnetic field from the alternating magnets of the form $\mathbf{B} = B_y = B_0 \cos(2\pi z/\lambda)$ then equation the transverse equation of motion becomes:

$$m\gamma \frac{dv_x}{dt} = e \frac{dz}{dt} B_0 \cos\left(\frac{2\pi z}{\lambda}\right) \quad (2.7)$$

Integrating both sides:

$$m\gamma v_x = \int e B_0 \cos\left(\frac{2\pi z}{\lambda}\right) dz \quad (2.8)$$

This leads to the transverse velocity:

$$m\gamma v_x = \left[\frac{eB_0\lambda}{2\pi mc} \right] \frac{c}{\gamma} \sin\left(\frac{2\pi z}{\lambda}\right) \quad (2.9)$$

The bracketed quantity is known as the deflection parameter, K :

$$K \equiv \frac{eB_0\lambda}{2\pi mc} = 0.9337 B_0 \lambda \quad (2.10)$$

with B_0 given in units of T and λ in cm. IDs with $K \gg 1$ are known as wigglers and produce radiation exhibiting a time-varying "search light" pattern with a broad spectrum [110]. IDs with $K \ll 1$ are known as undulators and have very small angular variance in their radiation and exhibit sharply peaked spectra. For these reasons, most modern ID beamlines use undulators to generate X-rays.

Ignoring the electron's energy loss while passing through an undulator, γ will be constant:

$$\begin{aligned} \frac{v_z^2}{c^2} &= 1 - \frac{1}{\gamma^2} - \frac{v_x^2}{c^2} \\ &= 1 - \frac{1}{\gamma^2} - \frac{K^2}{\gamma^2} \sin^2\left(\frac{2\pi z}{\lambda}\right) \end{aligned} \quad (2.11)$$

Or, after some rearrangement assuming a small K/γ :

$$\frac{v_z}{c} = 1 - \frac{1 + K^2/2}{2\gamma^2} + \frac{K^2}{4\gamma^2} \cos\left(2 \cdot \frac{2\pi z}{\lambda}\right) \quad (2.12)$$

Which contains an average velocity and a double-frequency term. The latter gives rise to interference pattern. In the time it takes the electron to travel one period (λ/v) the previous wavefront has propagated ($\lambda c/v$). So an observer at an angle θ from the $\hat{z}$ axis will see a path length difference of:

$$\delta = \frac{\lambda c}{v_z} - \lambda \cos(\theta) \quad (2.13)$$

Imposing of constructive interference condition:

$$\frac{\lambda c}{v_z} - \lambda \cos(\theta) = n\lambda_{rad} \quad (2.14)$$

with λ_{rad} the observed radiation wavelength. Taken with Equation 2.12 and assuming a small angle θ , one arrives at the well-known undulator equation:

$$\lambda_{rad} = \frac{\lambda}{2n\gamma^2} \left(1 + \frac{K^2}{2} + \gamma^2 \theta^2\right) \quad (2.15)$$

One can also consider the wavelength spacing between constructive and destructive interference. It can be shown that with N magnets comprising the undulator:

$$\Delta\lambda \sim \frac{\lambda}{Nn} \quad (2.16)$$

If one assumes a purely monochromatic source, it is also possible to find the destructive condition for θ that defines the divergence of the beam. It can be shown that:

$$\Delta\theta = \sqrt{\frac{2\lambda_{rad}}{N\lambda}} \quad (2.17)$$

More particularly, by Lorentz transforming classical dipole radiation from the electron's rest frame to the laboratory frame, it can be shown that the half-angle of the radiation cone produced, θ_{rad} is:

$$\theta_{rad} \approx \frac{1}{\gamma\sqrt{N}} \quad (2.18)$$

A critical result (Equation 2.15) is that the observed radiation $\lambda_{rad} \sim 1/\gamma^2$. For electrons at $\gamma = 10,000$, this corresponds to a reduction of 10^{-8} between the undulator's magnet spacing and the observed wavelength. In other words, centimeter periodicity in magnets can give rise to angstrom-scale radiation. The reduction has been critically exploited in the design of third and upcoming fourth generation synchrotron sources. Furthermore, Equations 2.16 and 2.18 make clear another feature of undulators: they produce relatively monochromatic and low-divergence X-ray beams. For these reasons, they are preferred at modern beamlines.

Bending magnets are devices designed to steer electron bunches around the storage ring. In doing so, they also produce radiation. A simplified description of a bending magnet is that it is an undulator with $N < 1$ total periods. Consequently, the bandwidth which goes as $1/N$ (Equation 2.16) gets very broad while the divergence, which goes as $1/\sqrt{N}$ (Equation 2.18) grows quite large. Often bending magnets are characterized by their critical photon energy E_c - the (laboratory frame) photon energy at which half the of the radiated power is above and half is below. The critical energy is given by [109]:

$$E_c = \frac{3e\hbar B\gamma^2}{2m} \quad (2.19)$$

The large divergence and broad bandwidth are undesirable for many X-ray experiments, especially elemental imaging, as they ultimately lead to reduced flux being delivered to the sample. With recent advances in beamline components, however, it is possible to use bending magnet beamlines to measure very dilute concentrations.

2.3.2 Focusing: KB mirrors, compound refracting lenses, and zone plates

Dating back to Röntgen's initial experiments with X-rays, it had been known that X-rays could not be readily focused with traditional lenses. The difficulty, it was later realized, lies in most materials having either real indices of refraction trivially different from 1 and/or because they are strong absorbers in the X-ray regime. Prior to the announcement of grazing-angle X-ray mirrors in 1947 by Ehrenberg [111] and their practical realization by Kirkpatrick and Baez [112], it was widely believed that X-rays could not be focused by mirrors or lenses. Widespread interest in microfocused X-ray experiments, however, pushed the development of several focusing mechanisms. This discussion is intended to be a brief introduction to each of these methods. In the interest of completeness, it is noted that capillary optics have also been used to focus X-ray beams [113–115] but are not discussed here.

The earliest reported focusing of X-ray beams was by so-called Kirkpatrick-Baez (KB) mirrors [112]. While significant advances have been made in the manufacturing of KB mirrors since their report 1948, the focusing principle remains the same. Two mirrors, one for horizontal and one for vertical focusing, are bent elliptically such that they have a common focal point. The geometry of focusing is similar to that of traditional mirror focusing and are not discussed here.

Compound refracting lenses (CRLs) use a series of holes bored through some material to effectively create a series of lenses. The index of refraction for materials in the X-ray regime can be written as

$$n = 1 - \delta + i\beta \quad (2.20)$$

with δ being the differential real index of refraction and β the absorption index. The trouble with forming traditional lenses lies in the fact that $\delta \approx 0$ for most materials with non-trivial absorptions. A CRL is a series of holes bored in a material with the area between holes acting as a series of lenses (Figure 2.4). With the focal length of a CRL is given by [116]

$$F = \frac{R}{2N\delta} \quad (2.21)$$

with R being the radius of the lens and N being the number of lenses. For example, aluminum at 10 keV has $\delta = 5 \times 10^{-6}$. Consider a single aluminum lens, $N = 1$, with a realizable radius of $R = 200 \mu\text{m}$. This lens' focal length is a 17.4 m - an impractical distance for many beamlines. By placing many lenses in serial, however, this distance quickly shortens. With 5 holes, for instance, the focal length becomes 3.5 m, a workable distance.

Figure 2.4. Schematic of a compound refracting lens (CRL).

Zone plates, in contrast, rely on diffraction to focus the incident X-ray beam. First described by Fresnel in 1866, a zone plate is a series of concentric rings with alternating transparent and opaque rings (Figure 2.5A) that focuses the incident beam. The geometry for the n th ring is shown in Figure 2.5B. For a source at point

A focusing at point **B**, the required radii are readily calculated by imposing the condition that the n th and $n + 2$ nd ring constructively interfere. The path length differences are given by:

$$\begin{aligned}\delta_1 &= \sqrt{r_n^2 + a^2} - a \\ \delta_2 &= \sqrt{r_n^2 + b^2} - b\end{aligned}\tag{2.22}$$

Figure 2.5. Schematic of a zone plate. **A.** Drawing of a zone plate, which consists of alternating dark and transparent layers concentric rings. The farther rings are from the center, the narrower rings have to be to achieve focusing. **B.** Figure illustrating the path length different for the n th ring.

rendering the total path length Δ :

$$\Delta = \delta_1 + \delta_2 = \sqrt{r_n^2 + a^2} + \sqrt{r_n^2 + b^2} - a - b\tag{2.23}$$

Imposing deconstructive interference requires that $\Delta = n\lambda/2$. After some rearrangement and assuming a small wavelength ($\lambda \ll r_n$), one arrives at the edge of the n th radius:

$$r_n^2 \approx n\lambda b + \frac{n^2\lambda^2}{4} \quad (2.24)$$

which gives the edge of the n th zone plate ring. The maximum attainable resolution of a zone plate, of course, is diffraction limited. Consequently, the Rayleigh criterion defines the maximum resolution. To resolve two objects a distance w apart:

$$w = 1.22\Delta r_{max} \quad (2.25)$$

Differentiating Equation 2.24 it is clear that zone thickness (Δr) must become thinner as n increases to achieve focusing. In practice, the fabrication of thinner rings limits the resolution attainable by zone plates.

Zone plates offer superior spatial resolution at the expense of considerably reduced flux, owing to their low diffraction efficiency ($\approx 20\%$). Modern focusing systems employ serial zone plates which, when aligned correctly, increase the diffraction efficiency. CRLs lie somewhere between the zone plates and KB mirrors as the entrance aperture must be comparable to the hole radius, R , which must be relatively small to achieve reasonable focal lengths. For X-ray images presented here, focusing was achieved by the method available at a given beamline, given in Table 2.3

2.3.3 Silicon drift detectors

Silicon drift detectors (SDD) are energy resolving detectors that are well suited for XRF experiments because of their high maximum count rate, energy resolution (~ 30 eV in modern SDD's) capable of resolving different elements, can be run at temperatures achieved by Peltier cooling, and their ability to subtend a relatively large solid angle [117]. The SDDs role as a detector has been developed around a scheme of sideways depletion, which was first proposed by Gatti and Rehak in 1984 [118]. Highly resistive n- type Si can be wholly depleted by contact with an n+ material provided that the contact is positively biased with respect to p+ contacts. In the depleted zones, the only free electrons will be a result of either radiation interacting

Table 2.3.
Focusing method used at each beamline

Beamline	Focusing Method	Achieveable Spot Size (μm)
ALS 10.3.2	KB Mirrors	3
2-ID-D	Zone Plate	0.15
2-ID-E	Zone Plate	0.25
8-BM	KB Mirrors	1.5 or 25
18-ID	KB Mirrors	5
18-ID	CRL	25 (h) $\times$ 2(v)
20-ID	KB Mirrors	7
21-ID	Zone Plate	0.03
26-ID	Zone Plate	0.04

with the Si wafer. The p+ contacts are arranged as a series of concentric circles whose voltages vary such that electrons are pushed towards an n+ diode in the center of the wafer [119]. The energy of the ionizing radiation can be determined by how many electrons were freed into the conduction band. By amplifying the collected electrons with a charge-sensitive amplifier, each event will correspond to a step in the recorded signal - the amplitude of that step is directly proportional to the energy of the ionizing photon, which allows energy resolution. This amplified signal is typically sent to a digitizer and filtering/pulse picking electronics before ultimately being converted into a list of energy-resolved photons.

There are several sources of spectral broadening and noise for SDDs to consider. Most notably is the full width at half maximum (FWHM) associated the detectors counting, which defines an intrinsic limitation of the resolution of a detector. Because the processes governing energy loss in the signal are not strictly independent, the

statistics are assumed to be a deviation from a Poisson distribution characterized by the Fano factor F . Under this distribution, the maximum attainable resolution is [109]

$$\Delta E_{Det} = 2.35\sqrt{F\epsilon E} \quad (2.26)$$

where E is the energy of incident bombarding photon, ϵ is the energy required to create an electron-hole pair and 2.35 is a conversion factor between standard deviation and FWHM for Poisson statistics. Thermally excited electrons being considered as signal is another major source of background for SDDs, which is minimized by cooling the Si element via the thermoelectric effect. Other sources of background, whose details are beyond the scope of this discussion, are electronic readout noise (especially associated with amplification), incomplete charge transfer as a result of trapping, and defects in the geometry of the silicon wafer.

In addition to the noise and peak broadening associated with the detector, there are several detector artifacts that must be considered when analyzing the spectra produced from an XRF measurement. Pile-up peaks, for example, occur when two separate photons are considered to be the same event that will have an effective energy of the sum of the two photons. While many detector systems have filters in place to detect and reject many pile-up events, inevitably some will be recorded. There are software techniques to correct for pile-up peaks. Another important artifact to consider is the Si escape peak. Si escape peaks occur when, instead of fully absorbing the incident X-ray, the Si wafer itself fluoresces. The effect of a Si escape event is that the observed peak is 1.74 keV (at the Si K_α line) lower than expected. This can also be accounted for in software while analyzing the spectra. Dead time corrections must also be taken into account when quantifying XRF experiments. Dead time refers to the time when the detector is not counting incident photons because it is busy recording, yet radiation is still incident on the detector freeing electrons. This can easily be accounted for by scaling the recorded counts by the live time.

In practice, there are several software packages available for fitting XRF spectra [120, 121] that take these issues into account. All work presented here was fit using the MAPS software package [120].

2.3.4 X-ray absorption near edge structure (XANES)

By taking small steps in incident energies through an element’s absorption edge, one obtains a XANES (X-ray Absorption Near Edge Structure) spectrum. XANES measurements provide an elementally specific means to probe local coordination environment and oxidation state. As thus, it has become an established technique in a broad range of fields including environmental, biological [122], and toxicological [123] communities. In it’s purest form, a XANES spectra are recorded by measuring the number of photons passing through a sample as a function of incident energy near the absorption edge. Above the edge, fewer photons will be transmitted through the sample. In practice, recording the fluorescence that results from absorption enables much more sensitive measurements.

X-ray absorption spectra can be split into three regions: pre-edge, the XANES region (near the absorption edge), and the extended x-ray absorption fine structure (XAFS) region (far beyond the edge). The energy of the absorption edge conveys the oxidation state of the element; more oxidized compounds have higher absorption energies. There are two interpretations of this edge shift. In one sense, core orbitals in oxidized species provide less shielding giving the nucleus a higher effective charge. From purely electrostatic considerations, then, it is sensible that the transition from p type to s type orbitals is energetically taxed when moving against the gradient of the higher effective charge. The oxidation sensitivity of XANES can also be considered from a geometric point of view. For XANES measurements, we are considering a transition between the core electrons (e.g. $1s$ for the K edge) to either continuum or unoccupied orbitals near continuum. Since higher oxidation leads to shorter bond lengths and the potential from the nucleus should behave as $1/r$, the edge energy must be higher for more oxidized species.

XANES data also provides information on the metal’s coordinating environment. If we assume only dipole ($E1$) transitions are allowed, then a transition to $1s$ from some metals valence d shell is forbidden. However, if the molecular d shell orbitals

exhibit p type characteristics (p - d hybridization) then the transition may occur, giving rise to a pre-edge. The pre-edge happens at a strictly lower energy than the K edge because it arises from a transition to an orbital that lies below continuum. While the edge energy is determined by the binding energy of the electron being sent to continuum, the location is also sensitive to the absorption site's binding energy. This can also be resolved through electrostatic arguments. An oxidized absorption site will have a higher effective charge as a result of weakened electronic shielding; consequently the electrons will be more tightly bound, increasing the energy gap between the K shell and continuum. The EXAFS region is not discussed here, but the curious reader is referred to [124].

2.4 Immunohistochemistry: a (very) brief primer

Immunohistochemistry (IHC) is a means to detect antigens, often proteins, in tissue sections. The labeling of specific antigens is possible due to antibody-antigen binding specificity. An immunoglobulin G (IgG) antibody is depicted schematically in Figure 2.6A. The antibody consists of a two identical large (heavy) and two identical smaller (light) polypeptide chains. At the end of each chain is the hypervariable region, depicted in orange, which is specific to the target antigen. Only immunoglobulin G (IgG) will be used in this work.

All of the immunostaining for this work is done with four basic incubations. First, tissue is fixed using paraformaldehyde (PFA). PFA is a long polymer (approaching $n = 100$) of formaldehyde which interacts with antigens to form crosslinks, thus preserving the structural integrity of the antigen. The aldehyde group can react with nitrogen in protein backbones and form a rigid methylene ($-\text{CH}_2-$) bridge between nearby reactive atoms. It is thought, then, that the sample is preserved by the rigidity of the matrix formed by methylene bridges. After fixation, the sample is incubated in a solution of bovine serum albumin (BSA) and Triton X-100. Triton X-100 is a common laboratory detergent used to permeabilize membranes. By extracting lipids, cell and

Figure 2.6. A: Schematic of an antibody. B: Schematic for IHC. The primary antibody binds to the desired antigen, and the secondary antibody binds to the primary antibody. Since the secondary antibody is fluorescently labeled, protein expression can be probed using this technique

organelle membranes become permeable to proteins and antibodies. Because the extraction takes place in the presence of polyoxyethylene moieties, there is no chemical specificity. Thus, permeabilization by Triton X-100 may give rise to false positives by removing proteins from cells; to ensure specific staining a negative control stain should be performed [125]. BSA is used as a blocking agent to prevent non-specific staining. While purified antibodies show preferential affinity for their target epitope, it remains a possibility that antibodies will bind to similar epitopes thereby producing false-positive fluorescence while imaging a specimen. Blocking serves to occupy all epitope binding sites with a protein that will a) still allow the higher affinity antibodies to bind, b) react with many epitopes in such a way that it does not change the epitope much, and c) does not react with the secondary antibody. By meeting these criteria, significantly fewer secondary antibodies will bind with undesired epitopes while the primary antibody may bind with its target and the fluorescent tag. After fixation and

permeabilization/blocking, samples are ready for incubation in primary and secondary antibodies (Figure 2.6B) and finally for imaging.

A table of antibodies used in this work is given in Table 2.4

2.5 Brain extraction and section preparation

This section outlines the standard protocol used throughout this work for animal sacrifice and section preparation. Details of animal treatment, where applicable, is given in the experimental description in later chapters. All experiments complied with animal rights regulations and were approved by the Institutional Committee on Animal Use at Purdue University and similar committees at collaborators' institutions.

With the exception of the cuprizone study, animals were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg) via intraperitoneal (IP) injection. Typically, the rodents are rendered unconscious within a few minutes as assessed by a tail pinch or eye tap. If necessary, additional injections were delivered. For the cuprizone study (Section 5.4), mice were anesthetized by inhalation of isoflurane. Once unresponsive, the iliac artery was severed and blood drained from the animal. The brain stem was then severed and the skull peeled away allowing the brain to be removed with a spatula onto a clean Kimwipe. After blotting the brain on the Kimwipe to remove excess blood from the surface, the whole brain was snap frozen in liquid nitrogen. Once frozen, the brain was placed on dry ice while other rodents were sacrificed before being moved for storage at -80 °C.

All tissue sections were cut on a Shandon FE/SE cryotome (Thermo-Fisher). The night before sectioning, unsectioned brains are moved from -80 °C storage to -20 °C and ultimately brought to -12 °C in the cryotome chamber. Brains (or bilaterally dissected brains) were mounted to the cryocassette using TissueTek Optimal Cutting Temperature (OCT) medium such that the medium never came in contact with the regions to be imaged. Sections were cut using a chilled BM-22 blade (Thermo Fisher)

at $\sim 2^\circ$ with respect to the sample head. To ensure correct sample alignment and position, sections were stained with cresyl violet and compared to a reference atlas (e.g. [126]). Samples 10-30 μm thick were taken on the following substrates:

- **Prolene[®] 4 μm thick polypropylene:** Compatible with XRF measurements. Polypropylene film was secured to plastic frames. Polypropylene is free of metals and shows no diffraction signal in the $d = 10 - 120 \text{ \AA}$ range. It does, however, exhibit a fiber diffraction pattern at $d \approx 4 \text{ \AA}$.
- **6 μm thick Mylar:** Compatible with XRF measurements. Mylar film was secured to plastic frames. Mylar is free of metals and shows no diffraction signal in the $d = 10 - 120 \text{ \AA}$ range or in the $d \approx 4 \text{ \AA}$ range.
- **Silicon nitride windows:** 50 nm thick Si_3N_4 windows. These are typically not used because they are fragile and expensive. However, both nanoprobe beamlines (21-ID and 26-ID at the Advanced Photon Source) have sample positioning systems that are designed around their use. Silicon nitride windows are compatible with both XRF and diffraction measurements.
- **Si wafers** 100 μm thick Si wafers were used for experiments in Section 4.1. These frames are designed to provide excellent thermal conductivity with the coldstage on Linkam cryostages.
- **Glass slides:** Standard glass microscopy slides for samples. These are not compatible with X-ray measurements but provide a convenient, flat substrate for optical microscopy.

Once thawed onto a substrate, samples are stored on dry ice while other sections are taken. Following a sectioning session, samples are stored at -80°C until use.

A map of typical sections taken is given in Figure 2.7.

Figure 2.7. **A.** Sagittal section from a rat brain Lateral 1.9 mm. The lines indicate the sections shown in B and C. **B.** Coronal rat brain section at Bregma - 0.8 mm. **C.** Coronal rat brain section at Bregma - 2.56 mm. Abbreviations are given in the list of abbreviations.

2.6 Statistical analysis

Throughout this work, except where noted, data are reported as the mean $\pm$ standard deviation. For comparison of groups, one-way analysis of variance (ANOVA) was used. Groups were considered statistically significant at the $p < 0.05$ level.

Table 2.4.
List of primary antibodies presented in this work.

Antibody	Abbreviation	Notes
Glial Fibrillary Acidic Protein	GFAP	Cytoskeletal marker for astrocytes
Ki67	Ki67	Nuclear marker for cells not in the resting S-State of cell cycle
Bromodeoxyuridine	BrdU	Nuclear marker for cells that have been created after BrdU injection
Frizzled 9	Fzd9	Nuclear marker for stem cells
Ubiquitin	UBQ	Marker for ubiquitin, which flags proteins for degradation
Lysosomal-associated membrane protein 1	LAMP1	Marker for lysosomes

3. Localization of Cu-rich aggregates in the rodent brain

Conventional methods for metal quantitation in the brain by atomic absorption spectroscopy, mass spectrometry or utilization of radioactive copper isotopes such as ^{67}Cu , lack fine spatial resolution. A recent review of neuroimaging techniques, for instance, listed laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) as having spatial resolutions ranging between 5-150 μm [127]. While such imaging is unquestionably valuable for brain research, it does not presently achieve resolutions required to explore phenomena on the sub-micron scale for which XRF is well suited. Secondary ion mass spectrometry (SIMS) has also proven to be a useful tool for biological imaging but has limitations related to a small probe volume. Numerous stains for copper have the required resolution but fail to provide quantitative information. Moreover, none of the conventional techniques can define the Cu oxidation state. Development of fluorescent sensors to Cu^+ is hindered by the properties of Cu^+ as a strong fluorescent quencher and the low stability of free Cu^+ in water medium. XRF imaging provides the sensitivity and spatial resolution necessary to reveal the distribution of metals in tissues and single cell. Furthermore, co-localization of XRF maps with histochemical markers allows for unambiguous determination of which cell types or organelles Cu aggregates reside.

Taking advantage of the excellent spatial resolution afforded by XRF, the localization of Cu aggregates in the SVZ may be determined. First, it will be demonstrated that elevated Cu in neurogenic areas is due to very intense, localized Cu aggregates of about 1 μm in size. These aggregates present in the subventricular (SVZ) zone of rats and mice. Furthermore, similar aggregates are demonstrated in the rostral migratory stream (RMS) and dentate gyrus of rats, but not mice. While the origin of this species discrepancy is unknown, it may be related to the ability of rats to produce more neurons and have them functionally integrated more readily. The SVZ is

composed primarily of four cell types (Section 1.5), and it will be shown by combined XRF and IHC that Cu aggregates reside specifically in type-B cells. Finally, seeking to determine if aggregates are present in autophagic processes, it is shown that Cu aggregates do not reside in lysosomes and are not immunoreactive to ubiquitin. As these represent the two major pathways of autophagy, the data suggest that Cu aggregates play some role and are not merely awaiting degradation.

3.1 Cu-rich inclusions are found in cells in neuroproliferative zones

Previous reports have found the neurogenic periventricular regions [105,128] and dentate gyrus [104] of rodents to have elevated Cu. These studies, by LA-ICP-MS, could not achieve the required spatial resolution to distinguish between elevated homogenous Cu distributions and discrete puncta. With submicron resolved XRF imaging, it is possible to resolve individual Cu granules, confirming that Cu in neurogenic niches is present as inclusions. Table 3.3 summarizes where XRF microscopy has determined Cu aggregates to be present. Both rats and mice exhibit Cu aggregates in the subventricular zone. As neurogenic activity extends from the SVZ along the rostral migratory stream (RMS) *en route* to the olfactory bulb (Figure 2.7), it was of interest to determine if Cu-rich aggregates also exist along the RMS. The rodent RMS has been reported to be supported by an astrocytic ribbon made of type-B cells [129,130]. If aggregate containing cells themselves migrate along the RMS, one expects to find aggregates along the RMS. It was found that rats, but not mice, have Cu aggregates along the RMS. While the cause of this difference is not yet clear, it highlights the importance of species consideration when studying neurogenesis in the SVZ and RMS. The final neurogenic niche – the dentate gyrus (DG) of the hippocampus which is much better characterized than the RMS – was also examined. Similar to the RMS it is shown that rats but not mice have Cu aggregates. These results suggest the role of Cu may be related to neuronal production and integration.

3.1.1 Experimental details

The primary aim of this section is to demonstrate that previously reported elevated Cu concentrations in neuroproliferative zones is due primarily to Cu inclusions, rather than as a basal cellular Cu concentration. As such, it is a summary of a variety of species imaged over several years. XRF imaging conditions are given in Appendix A from sections prepared following protocol described in Section 2.5.

As the majority of Cu in the SVZ and RMS (for rats) lies in a single cell layer, it can be difficult to objectively choose a region of interest with 25 μm resolution. Concentrations in Table 3.2 are the result of analyzing 25 μm images (e.g. Figure 3.3A,B). To mitigate the influence of the experimenter's choice, ROIs for analysis were chosen as follows: a line was drawn to select either the SVZ or RMS. This line was extended by 3 pixels on either side perpendicular to the line making the total ROI 7 pixels (175 μm) wide. It should be noted that this will artificially lower the reported Cu concentration. It is possible to use concentration thresholding to isolate Cu hotspots within the wide region. However, the vast differences in Cu concentration between rats and mice make that a complicated issue and unthresholded data serves for the purposes of this discussion. The striatum and cortex are large, homogenous areas so their mean concentration was determined by hand drawn ROI selection.

To supplement XRF images in the dentate gyrus, dark field micrographs of 3,3'-diaminobenzidine (DAB) stained hippocampi are included (images provided by Dr. John Young, Howard University). DAB is a compound that forms a dark precipitate in the presence of peroxidase or pseudoperoxidase activity. DAB staining was done by incubating sections in a DAB mixture (25 mg of DAB were added to 50 ml of 0.05 M Tris buffer, pH 7.2, and then mixed with 1 ml of 3% H_2O_2) for 30 minutes following 3 $\times$ 5-minute PBS rinses. DAB staining in the SVZ is presented and co-localized with XRF maps in Section 4.3. It should be noted that only a subset of DAB+ granules represent Cu-rich aggregates.

3.1.2 The distribution of Cu aggregates in rats and mice in neuroproliferative zones

Figure 3.1 shows Cu XRF fluorescence maps recorded from rat brain sections. In rat brain, Cu accumulations were detected along the ventricle walls, in the external capsula, and in the septofimbrial nucleus and triangular nuclei. In particular, a considerably elevated Cu content was noted along the lateral ventricle wall [128]. Similar distributions are observed in the SVZ of mice (Figure 3.3, Table 3.2).

Figure 3.1. Cu XRF maps from coronal rat brain sections at Bregma = -0.8 mm (A) and Bregma - 2.56 mm (B)

To determine if accumulations are intracellular, which the local fluctuations in intensity suggest, brains were imaged with 300 nm resolution. XRF microscopy with subcellular resolution on rodent brains revealed Cu accumulations in cells of the SVZ (Figure 3.2). Additionally, XRF microscopy provides elemental markers of the cell structures. For instance, cell nuclei were identified by increased phosphorous signals, while sulfur and iron were distributed throughout the cytoplasm. Potassium was concentrated inside the cell, thus helping to define the overall cell shape. XRF mi-

Table 3.1.
Cu Concentrations of Cu-rich inclusions in the SVZ

Rodent	Area Number	[Cu] (mM)
Rat 1	1	237.62 ± 81.8
Rat 1	2	203.00 ± 91.2
Rat 2	1	313.16 ± 113.3
Rat 3	1	168.38 ± 62.9
Rat 3	2	240.77 ± 95.9
Rat 3	3	341.48 ± 135.3
Mouse 1	1	368.24 ± 228.1
Mouse 2	1	398.13 ± 173.1

croscopy clearly showed Cu rich accumulations up to 1 micron in size and with Cu concentrations on the order of hundreds of mM (Table 3.1). Cu accumulations were detected in close proximity to cell nucleus (as visualized by increased phosphorus signal) as well as at considerable (up to 5 μm) distances from the nucleus. Remarkably, the detected Cu rich accumulations had low concentration of other metals such as Zn and Fe (Figure 3.2). A lack of co-localization with Zn eliminates the possibility that they originate from Cu/Zn superoxide dismutase 1. Co-localization of the Cu signal with S was prominent in all positions of increased Cu content (Figure 3.2, Table 4.1). This distribution was observed in both rats and mice.

Analysis of Cu in the SVZ and RMS of 12 week old Sprague–Dawley rats and CD-1 mice (Table 3.2) reveal some striking differences. First, Cu concentrations in the SVZ are nearly $2.5\times$ higher for mice than rats. By contrast, the striatum and cortex – regions where Cu-rich aggregates are not observed – are only $1.7\times$ and $1.3\times$ higher. Secondly, in rats the RMS has a Cu level comparable to the SVZ and considerably above the striatum and cortex, as is evident in XRF maps (Figure 3.3A). Mice, in contrast, have a mean RMS Cu concentration well below that of the SVZ and show

Figure 3.2. Cu inclusions in the SVZ. **A.** XRF microscopy in mouse brain clearly demonstrates intracellular structures with dramatically increased Cu content. The cell nucleus is identified by the increased phosphorous signal, while sulfur is distributed throughout the cytoplasm. Potassium has much higher concentration inside the cell than outside and, thus, helps to define the overall cell shape. **B.** XRF image of the SVZ. Elemental concentrations are in $\mu\text{g} / \text{cm}^2$ in 10 μm thick sections. The dashed line shows the ventricle wall with brain tissue in the right low corner. From XRF imaging it is evident that SVZ selectively accumulates Cu over other trace elements. **C.** XRF rat brain recorded with increased resolution along the ventricle wall showing that the Cu is also present in rats as intracellular aggregates. Cu K_{α} fluorescence is shown in red while Compton scattering is in cyan. The white dashed line indicates the ventricle wall. **D** Image of the same section as (C) after XRF analysis at room temperature stained with cresyl violet demonstrates tissue integrity after exposure to X-rays.

Table 3.2.
Copper concentrations in the SVZ and RMS

	12 week Sprague–Dawley Rat	12 week CD-1 Mice
<i>n</i>	5	3
SVZ	8.58 ± 1.03	24.45 ± 1.21
RMS	7.82 ± 2.02	8.93 ± 0.60
Striatum	6.30 ± 1.52	10.81 ± 2.12
Cortex	6.00 ± 1.35	7.88 ± 0.18

no contrast between the cortex or striatum (Figure 3.3B). Higher magnification XRF maps show that the rat RMS elevated Cu is due to Cu-rich aggregates (Figure 3.3C) whereas mice do not exhibit any contrast along the RMS (Figure 3.3D).

Cu concentrations in the RMS have not been previously reported. Here it is demonstrated by XRF microscopy that Cu concentrations in the RMS are comparable to those of the SVZ (Table 5.3) for rats but not mice. At present, there is no firm explanation for this. Cu that forms accumulations must arrive at the RMS either through direct uptake or via formation in the SVZ followed by migration through the RMS.¹ In the case of the former, these results suggest that Cu uptake is fundamentally different between mice and rats, at least in the RMS. As CTR1 and DMT1 are thought to be the primary Cu transporters across brain barriers (Section 1.2), these would be prime candidates. CTR1 is known to be expressed at both the blood-brain barrier and blood-cerebrospinal fluid barrier whereas DMT1 is only suspected along the blood-brain barrier [35]; it is possible that rat astrocytes have enhanced Cu uptake compared to mice resulting in the RMS accumulations. The latter would suggest that cells in rats and mice have very different migratory behavior along the RMS.

¹It is also possible cells in the mouse RMS have some ability to remove these accumulations that rats do not possess. However, there is no clear mechanism for that.

Figure 3.3. Cu XRF maps of **A.** rat sagittal section and **B.** mouse sagittal section with 25 μm resolution. While Cu concentrations are higher in the SVZ for mice, Cu aggregates do not exist along the RMS. **C.** Cu XRF map of a rat RMS with 1.5 μm spatial resolution, showing that Cu is present as aggregates in the RMS. **D.** Cu XRF map of a mouse RMS with 10 μm resolution showing that there are no Cu-rich aggregates along the mouse RMS. Display range is given in $\mu\text{g/g}$ in 30 μm sections.

In the dentate gyrus of the hippocampus, rats showed both DAB-staining cells (Figure 3.4C) and Cu accumulations detected using X-ray microscopy (Figure 3.4D).

Mice of all strains surveyed (Table 3.3) harbored no Cu-rich hippocampal accumulations and did not exhibit DAB+ staining (Figure 3.4A,B).

Figure 3.4. Distribution of Cu aggregates in the hippocampus. **A.** Dark field micrograph of a DAB-stained mouse section shows no aggregates. **B.** X-ray fluorescence maps of a mouse hippocampus also shows no Cu accumulations, confirming that the Cu aggregates present in rats and in the SVZ of both species are not present. **C.** Dark field micrograph of a DAB-stained rat section reveals positively-stained aggregates in the dentate gyrus. **D.** Similarly, elemental maps show dense Cu-inclusions in dentate gyrus of the rat hippocampus. Scale bars: 100 μm . Display ranges are in mM through a 10 μm thick section.

As to the functional role of inclusions, one expects the species difference in Cu aggregate presence to manifest itself somehow. One possible manifestation is that mice show reduced hippocampal neurogenesis and circuit integration in comparison with rats. For example, newly born neurons in the dentate gyrus are three-fold more numerous in adult rats as compared to adult mice, and are ten times more likely to be recruited into learning circuits [131]. A diminished number of neurogenic astrocytes in mice, as well as reduced oxidative stress, may well be the explanation for the species difference we observed. Other species differences in the rat and mouse hippocampus have been reported. In the mouse, dentate gyrus neurons are immunoreactive for

Table 3.3.
Species summary of Cu inclusions in Neuroproliferative Zones

Species	SVZ Cu Inclusions	RMS Cu Inclusions	DG Cu Inclusions
Sprague-Dawley Rat	Cu+	Cu+	Cu+
CD-1 Mouse	Cu+	Cu-	Cu-
C57BL/6J×SvImJ Mouse	Cu+	Cu-	Cu-
129S1/SvImJ Mouse	Cu+	Cu-	Cu-

calretinin, but in the human and rat, these neurons fail to stain for this calcium-binding protein [132, 133]. Also, cholecystokinin immunoreactivity can be found in the mouse dentate gyrus, but not in the rat. Finally, dentate gyrus neurons of the mouse fails to stain for calcitonin gene-related peptide, but those of the rat show prominent staining [132].

The difference between rats and mice conveys that there may be critically different mechanisms at play with regard to neurogenesis and/or migration. These differences will be important to consider moving forward, especially as literature in the field often lumps results from rats and mice together.

3.2 Cu-rich inclusions reside in glial-fibrillary acidic protein-positive (GFAP+) cells

A critical step in determining the role of Cu aggregates is to determine in which cell types they reside. The existence of these aggregates in a particular cell type implicates a function that coincides with that cell type's function; on the other hand, the presence of aggregates in all cell types would suggest that inclusions have more universal role in the region and may be spread through cell division. To that effect, two attempts were made to determine the cell specificity of Cu-rich aggregates in the SVZ. First, cells were labeled for glial fibrillary acidic protein (GFAP) using

gold nanoparticles for XRF imaging. As gold is not present in detectable levels in healthy brains, any detected gold must be a marker for GFAP. Co-localization of GFAP and Cu aggregates demonstrates that Cu resides in GFAP+ cells. Secondly, co-localization of optically detected GFAP Cu X-ray maps confirms that aggregates reside in GFAP+ cells. It is demonstrated that Cu aggregates reside in GFAP+ cells. Furthermore, aggregates do not co-localize with Br fluorescence corresponding to bromodeoxyuridine (BrdU) positive cells – cells that have been born or divided following BrdU treatment – suggesting that the inclusions reside in slow-proliferating type-B cells.

3.2.1 Experimental details

Male Sprague–Dawley rats (Harlan, Indianapolis, IN.) were purchased 7-8 weeks old and were housed in a temperature-controlled, 12/12 light/dark room, and allowed free access to pelleted rat chow (Purina rodent chow 5001, 13 ppm Cu content) and distilled, deionized water. At the age of 12 weeks, the rats were sacrificed and sections were taken following the procedure in Section 2.5.

For immunolabeling with GFAP, sections were thawed, fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline for 30 minutes at room temperature (RT), washed twice with PBS, treated for 1 hour at RT sequentially with: 1) blocking/permeabilization solution: (2% (w/v) bovine serum albumin (BSA) / 0.2% (v/v) Triton X-100 in PBS), 2) primary antibodies in PBS containing 2% BSA with 1:200 rabbit anti-GFAP (abcam ab15580), 3) secondary antibodies in PBS containing 2% BSA: either Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen A-11034)) at 1:1000 dilution for optical microscopy or Nanogold 1.4 nm anti-rabbit IgG Au nanoparticles (Nanoprobes #2003) 1:120 dilution for XRF microscopy. Three, five minute washes with PBS were performed after treatment with primary and secondary antibodies. Sections on microscope slides were subjected to ProLong Gold antifade reagent before being covered with a coverslip. Slides were sealed and stored at 4°C. Sections

for XRF were quickly rinsed with ultrapure water to remove excess P and Cl from residual PBS.

BrdU, a cell proliferation specific marker, can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of cell cycle) in place of thymidine. BrdU was dissolved in saline to final concentration 10 mg per 1 ml. The solution of BrdU was prepared twice in a day and kept on ice. 2 rats 12 weeks old, 350 and 330 g, received 45 mg BrdU daily via five IP injections for 6 consecutive days. Before sacrificing the rats were not injected for 24 hours.

XRF imaging was done at beamline 2-ID-D at the Advanced Photon Source with an incident energy of 12.0 keV for Au-GFAP co-localization, 10.0 keV for optical GFAP co-localization and 13.8 keV for BrdU detection. Au was quantified by L edge fluorescence. The pixel size for co-localization experiments was 0.3 μm .

3.2.2 Results of GFAP/Cu aggregate co-localization

XRF-microscopy with subcellular resolution demonstrated that not all cells in the SVZ contained the Cu enriched accumulations (Figure 3.5). This is due to the heterogeneous cellular composition of the SVZ with four types of cells (Section 1.5): ependymal, GFAP-expressing astrocytes - type B progenitors, type C transit amplifying cells and type A migrating neuroblasts, comprising about 95% of its cell population [83, 85, 134]. Co-localization of Cu XRF with optical and XRF-IHC fluorescence of immunochemically stained glial fibrillary acidic protein (GFAP), a marker of GFAP expressing astrocytes, demonstrated that Cu is preferentially localized in the GFAP positive cells (Figure 3.5A,B). For Cu occurring in cell bodies we observed prominent GFAP/Cu co-localization due to bright GFAP stain of the cell bodies. Weaker GFAP staining of processes also shows co-localization with Cu when GFAP is visualized at decreased dark level. These results are in agreement with Cu / P distributions showing Cu accumulations at significant distances from cell nucleus (Figure 3.5A,B).

Figure 3.5. **A.** Co-localization of optical fluorescence of immunochemically stained GFAP (green) and X-ray fluorescence from Cu (red). The white dashed line indicates the ventricle wall. **B.** Subcellular co-localization of Cu with GFAP as visualized by immunostaining with gold nanoparticles as a secondary antibody. **C.** Left: Schematic of the cellular architecture of the SVZ. Progenitor cells (A-C) in the SVZ lie adjacent to the ependymal cell (E) layer lining the lateral ventricles. Right: XRF image of cells located in the SVZ of a Sprague-Dawley rat treated with BrdU a marker of actively dividing cells. BrdU identifies type A cells - migrating neuroblasts and type C cells - transit amplifying cells. Co-localization of Br and phosphorus X-ray fluorescence signals shows actively dividing cells (dashed circles). Areas occupied by dividing cells have a diminished number of Cu-accumulations. The black arrows indicate the ventricle wall.

Injection of rats with the proliferation marker BrdU allowed us to identify actively dividing cells of type A and C (Figure 3.5C). In the brains of these rats, XRF measurements above the energy of the Br K edge detected the distribution of Br, while measurements in control rats did not detect any Br signal. Selective visualization of nuclei with Br enrichment was done by co-localization of Br and phosphorus signals (Figure 3.5C). Moreover, the cells with Br-enriched nucleus were smaller than the others. This is in agreement with earlier studies showing that A-type cells are smaller in size (about 8 μm) than those of the B- or C-type [83]. A-type cells are also elongated along the direction of their migration along the ventricle wall (this direction is perpendicular to the plane of the image). While areas occupied by actively dividing cells were mostly free from Cu accumulations, the cells surrounding them showed Cu signals. It is known that type B progenitors surround actively dividing cells of the SVZ (Figure 3.5C). These data thus support the notion that Cu primarily accumulates in type B progenitors.

The exclusivity of Cu inclusions to astrocytes may be elucidating for its transport mechanism into the brain. Like any mammalian cells, astrocytes uptake Cu into the cytoplasm by copper transporter 1 (Ctr1). Ctr1 passes Cu to Cu chaperones, which mediate the intracellular transport of Cu to cuproproteins via mechanisms utilizing direct protein-protein interaction. Possible pathways for the Cu transport into the astrocytes include Cu absorption either from the blood or the cerebrospinal fluid (CSF). The BBB is partially lost on the surface of blood capillaries in the SVZ [135] which can explain highest Cu accumulations detected in astrocytes positioned close to the ventricle wall. The choroid plexus, a tissue in brain ventricles adjunct to the SVZ, transports Cu and other metals between the blood and the CSF [136]. The CSF is separated from the cells of the SVZ by only one layer of ependymal cells, which do not form tight junctions. Some of the type B progenitors retain direct exposure to the surface of the ventricle wall even in adults [48]. Thus, astrocytes have preferential (as compared to neurons) access to the interstitial fluids as well as the CSF, in part due to their perivascular endfeet. Observation of the extensive Cu accumulations in the

astrocytes of the SVZ and partially in the ependymal cells might lead to the idea that cells in this part of the brain function as an additional barrier between systemic fluids and brain tissues (at least in terms of Cu transport). In such a case, accumulation of Cu can be envisioned as a result of Cu retention at this barrier. However, it is an unlikely function and that the astrocytes are rather balancing the Cu content in the brain to meet the brain's Cu needs than simply achieving Cu detoxification. In support of such a view speaks the observation that the local Cu concentration in the astrocytes is several orders of magnitude higher than those in the blood and CSF (e.g. Table 5.1) which would imply a facilitated Cu uptake and retention. Moreover, the concentrations of other possibly toxic metals such as Zn, Mn and Fe are not increased in these cells as compared with brain cells at other locations, showing that other metal ions are not accumulated at this potential barrier. This therefore implies that astrocytes are uniquely capable of absorbing and retaining Cu ions. The ability of astrocytes to retain Cu might also relate to the fact that these cells do not express the ATP7B Cu transporter responsible for Cu export from the cell [137].

3.3 Cu-rich inclusions are not found in lysosomes or ubiquitinated

One hypothesis for the origin of these accumulations is that they are vestiges of some other cellular process. If this were the case, it is reasonable to speculate that they will be deteriorated at some point. There are two major cellular autophagic pathways. Lysosomes are the workhorse of macroautophagy giving rise to the degradation of the autophagosome it fuses with and its contents. More recently, attention has been given to proteosomal degradation, particularly the ubiquitin-proteasome system (UPS, see [138] for a review). To determine if accumulations were involved in a derogatory pathway, co-localization experiments were carried out for Cu accumulations against lysosomal-associated membrane protein 1 (LAMP1) and ubiquitin (UBQ).

3.3.1 Experimental description

LAMP1 staining was done on a section from a 9 week old 129/SvImJ mouse and UBQ staining on a 12 week old Sprague–Dawley rat. Sections were prepared according to the protocol in section 2.5. For immunolabeling with LAMP1/UBQ, sections were thawed, fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline for 5 minutes at room temperature (RT), washed three times with PBS, treated for 2 hour at RT sequentially with: 1) blocking/permeabilization solution: (2% (w/v) bovine serum albumin (BSA)/0.2% (v/v) Triton X-100 in PBS), 2) primary antibodies in PBS containing 2% BSA with either 1:200 rabbit anti-LAMP1 (abcam ab24170) or 1:50 rabbit anti-ubiquitin (abcam ab7780), 3) secondary antibodies in PBS containing 2% BSA: either Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen A-11034)) at 1:1000 dilution for optical microscopy or Nanogold 1.4nm anti-rabbit IgG Au nanoparticles (Nanoprobes #2003) 1:120 dilution for XRF microscopy. Three, five minute washes with PBS were performed after treatment with primary and secondary antibodies. Sections on microscope slides were subjected to ProLong Gold antifade reagent before being covered with a coverslip. Slides were sealed and stored at 4 °C. Sections for XRF were quickly rinsed with ultrapure water to remove excess P and Cl for PBS.

XRF imaging was carried out at beamline 2-ID-D (LAMP1) or 2-ID-E (UBQ) of the Advanced Photon Source at an energy of 13 keV and a pixel size of 0.3 μm . Au was detected by its L fluorescence.

3.3.2 Results of Cu-autophagic marker co-localization

Figure 3.6 shows immunostaining for the autophagic markers. Au labeled XRF maps are shown on the right (Figures 3.6 B,D) and optical stains of comparable sections are shown on the left (3.6 A,C) for comparison. It is clear that Cu inclusions do not co-localize with lysosomes (via LAMP1 staining) and are not ubiquitinated, which would be required for proteosomal recognition. These data suggest that Cu aggregates are not involved in an autophagic process.

Figure 3.6. Co-localization of Cu inclusions with lysosomes (LAMP1) and ubiquitin (UBQ). **A.** Optical micrograph of LAMP1 staining showing that LAMP1 stains small puncta that reside mostly in cells, including GFAP+ astrocytes. **B.** XRF co-localization of Cu (green), S (red), and Au-labeled LAMP1 (blue) showing that Cu does not reside in lysosomes in the SVZ. **C.** Optical micrograph of ubiquitin staining in the SVZ. Ubiquitin also forms small puncta that reside in cells. **D.** XRF co-localization of Cu (green), S (red), and Au-labeled ubiquitin (blue). These data show that Cu inclusions are not ubiquitinated.

The non-involvement in autophagy is elucidating for the role of the Cu aggregates in the cell. Firstly, it dismisses the possibility that the aggregates are a part of aggresomes. An aggresome is a pericentrosomal, microtubule-dependent collection of misfolded proteins flagged located [139, 140]. If Cu bodies were active in the UPS, they would be immunoreactive to ubiquitin [141], which they are not (Figure 3.6D). It will be shown in Section 5.1 that Cu aggregates become more numerous with age. It is possible that the aggregates, in part, permit a mechanism of toxicity of these inclusions at advanced age. In human embryonic kidney cells, for instance, the presence of protein aggregates has been shown to compromise the UPS, either through interaction with the proteasome itself or an auxiliary protein [142]. This leads to a feedback loop in which individual proteins cannot be degraded quickly enough and thus can form aggregates, the product which may not be immunologically active for ubiquitin. While cells are prone to aggregation when treated with proteasome inhibitor [143–145], cultured astrocytes in particular remain resilient due to their ability to upregulate macroautophagy under proteosomal stress [146]. While more-active macroautophagy protects cultured astrocytes, the *in vivo* fact is that advanced age comes with diminished ability to carry out macroautophagy [147]. With an impaired UPS and macroautophagy, older brains will be unable to correctly handle misfolded proteins, which may ultimately lead to neurodegeneration.

4. The composition of Cu-rich aggregates in the brain

Just as knowledge of where aggregates are located in the brain is critical, so too is characterization of the composition of aggregates. Unbound Cu is cytotoxic as it induces an avalanche of oxidative species that can lead to cell death. As such, it is expected that hundred millimolar Cu aggregates will be bound in some protein environment. To ascertain the traits of the binding environment, X-ray absorption near edge structure (XANES) spectra were recorded directly from tissue. Obtained spectra indicate that Cu is bound as Cu^+ and is present in a Cu_xS_y multimetallic cluster. Consistently, elemental analysis from XRF imaging with 30 nm resolution demonstrates that Cu and S consistently co-localize with a molar ratio $[\text{S}]/[\text{Cu}] = 1.66 \pm 0.07$. Furthermore, Fe and Cu are shown to form separate, peroxidase (or pseudoperoxidase) activity exhibiting aggregates in the SVZ, which clarifies previous work characterizing Gomori-positive aggregates. These results are consistent with assignment of metallothionein as the binding protein, but are not sufficient to unambiguously determine the protein. Finally, considering many metal containing aggregates form via a tau-facilitated method, X-ray diffraction is recorded directly from Cu aggregates. It is shown that while aggregates exhibit Bragg peaks, they do not exhibit the hallmark cross- β motif of other metal aggregates and so there may be a new metal-related aggregation mechanism involved in aggregates' formation.

4.1 Cu in aggregates is present as Cu(I)

Determining the oxidation state and coordinating environment of Cu in Cu aggregates is critical to assigning a binding protein and elucidating their function. X-ray absorption near-edge structure (XANES) is uniquely sensitive to metal oxidation states and can furthermore be combined with microbeam imaging to ensure that Cu

inclusions are measured instead of bulk brain Cu oxidation state. Cu aggregates from thin (10-30 μm) brain sections were located with XRF microscopy and subsequently probed with XANES measurements. These measurements reveal that Cu is present in inclusion as Cu^+ and as a Cu_xS_y multimetallic cluster.

4.1.1 Experimental description

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) were purchased 7-8 weeks old and were housed in a temperature-controlled, 12/12 light/dark room, and allowed free access to pelleted rat chow (Purina rodent chow 5001, 13 ppm Cu content) and distilled, deionized water and sacrificed at 12 weeks old. Sections at Bregma - 0.8 mm were thawed onto 100 μm thick Si wafers and stored at -80 °C until measurement as described in Section 2.5.

For recording XANES directly from tissue, the sample must be kept very cold to mitigate X-ray induced damage. To achieve this, a Linkam cryostage with a programmable temperature controller unit was mounted on the motorized stage at ALS beamline 10.3.2 [148] and set to 80 K. Measurements were taken with a beam size of 3 μm $\times$ 3 μm . As Cu inclusions constitute the majority of Cu in any individual sampling voxel, it is assumed that recorded XANES reflects the oxidation and coordination state of aggregated Cu. Brain samples on 100 μm thick Si wafers, designed for optimal thermal conductivity and tight contact with the silver cold block of the cryostage, were placed on the cryostage via the sample port to prevent thawing. For energy calibration of the Cu K-edge XANES, Cu foil was used with $E_0 = 8980$ eV, while internal scan-to-scan calibration was done using a monochromator glitch.

To compare recorded XANES from brain tissue with Cu-metallothionein, a suitable reference compound for Cu_xS_y compounds, Cu_6 –metallothionein(I_I) was prepared as follows [149]. Apo-Metallothionein (1A/2B mixture) was purchased from Bestenbalt LLC (#031-2000) and dissolved to 1.6 mM in Tris-HCl buffer, pH 7.8. To reduce oxidized apo-MT, dithiothreitol (DTT) was added at a concentration of

48 mM (ratio DTT:MT = 30) and the mixture was incubated for 1 hour at RT. To remove excess DTT, 100 μ L of apo-MT and DTT mixture was loaded into a small G-25 sephadex column, equilibrated with 0.01 HCl, and 1.3 ml of reduced apo-MT was collected (final concentration $\approx$ 0.12 mM). CuCl at 10 mM was prepared in a solution of 0.1N HCl and 4% NaCl and added to reduced apo-MT at ratio 6:1. The mixture was incubated for 30 minutes at RT. After incubation, pH was adjusted with Tris/HCl to pH 7.8. Cu⁺-bound MT was concentrated using an Amicon Ultra-4, PLBC Ultracel-3 Membrane, 3 kDa centrifugal filter unit (Millipore, UFC800324) up to $\approx$ 3 mM. Holders for XAS were filled with the concentrated sample and placed immediately in a -80 °C freezer.

4.1.2 Results of Cu-XANES in the rat SVZ

Information on the copper's oxidation state and ligand environment is critical to elucidate the mode of its intracellular binding and its biological role in the cell. Cu⁺ ions at the Cu K-edge are known to exhibit an intense feature at $\sim$ 8983 eV corresponding to an electric dipole-allowed $1s$ -to- $4p$ transition. The intensity of this transition is sensitive to the occupancy of the Cu $4p$ orbitals which differs according to the coordination (i.e. diagonal, trigonal, tetragonal, etc.). The shape and intensity of this feature can be used to determine the coordination environment of the Cu⁺ site in proteins [150,151]. XANES on the intracellular Cu rich accumulations was recorded at low (80 K) temperature ensuring non-damaging conditions (Figure 4.1). Spectra obtained from different Cu enriched accumulations were the same within signal-to-noise (Figure 4.1C). This demonstrated that Cu inside different accumulations has the same binding environment. The energy of the Cu K-edge unambiguously identified the Cu oxidation state as Cu⁺. A comparison of the obtained XANES spectrum with spectra of model Cu compounds and Cu bound proteins available in the literature showed that it was characteristic for the cuprous-thiolate multimetallic cluster and closely resembled the spectra published for a variety of Cu-metallothioneins. The

work of Pickering *et al.* [151] illustrates that details of Cu XANES spectra are different for different proteins containing cuprous-thiolate multimetallic clusters. This is not surprising as the electronic structure of the metal clusters in proteins is known to be affected by the protein environment. In Cu XANES the number of transitions and their relative intensities are sensitive to the electronic structure of the Cu_xS_y cluster. Assignment to cuprous-thiolate multimetallic cluster agrees with the identification of a predominantly sulfur coordination environment from co-localization of the Cu and S signals from XRF microscopy (Section 4.2). Comparison of the Cu XANES spectra from the intracellular compartments with the spectrum of Cu-MT prepared from rabbit apo-metallothionein and recorded at the same experimental conditions demonstrated a high degree of similarity (Figure 4.1D).

Taking into account how dangerous elevated Cu levels can be better understanding of Cu-binding/sequestration in astrocytes is critical. Intracellular Cu K-edge XANES measurements are instrumental in this regard. The high degree of similarity between the XANES spectra of intracellular Cu in astrocytes and those of prepared Cu-MT suggests that Cu is bound to MT or protein providing similar Cu binding environment with the formation of multimetallic Cu_xS_y cluster. One of the hallmarks of a multimetallic Cu^+-S cluster XANES spectrum a monotonic increase of absorbance with energy near the Cu K edge (8979 eV) due to the Cu-Cu binding. There are numerous examples in the literature [152,153] which compare Cu-S clusters to model compounds with only one Cu that exhibit decreased absorbance immediately following the edge. Since spectra from both tissue and Cu-MT exhibit increasing absorbance at the edge, it is concluded that the Cu is bound in the form of multimetallic cluster. Furthermore, Table 4.1 illustrate that the $[\text{S}]/[\text{Cu}]$ ratio is comparable to the one expected for Cu-MT. High local Cu concentrations (100-500 mM) indicates potential aggregates and MT has been shown to form aggregates with Cu in pathological conditions [154]. In the brain, astrocytes are the principal source of MT I and II, although other cell types also express MT I and II [155,156]. Astrocytes of the SVZ show intense MT I, II expression detected by *in situ* hybridization and immunohistochemistry [157,158].

Figure 4.1. **A.** Schematic diagram of coronal section located at approximately Bregma -0.8 mm. The red box indicates the approximate area of the XRF image. **B.** XRF image of copper distribution demonstrates that Cu enrichment is localized to a thin layer along the lateral ventricle wall. XANES measurements (**C**) were taken on Cu accumulations, indicated by arrows. Spectra obtained from different Cu-accumulations are the same within signal-to-noise. The red line is the average of all XANES measurements taken on this sample. **D.** Intracellular Cu K-edge XANES spectrum at low temperature on the Cu accumulations in a rodent brain (red) recorded at the same experimental conditions as the spectrum of Cu-MT prepared from rabbit apo-MT (blue). The high degree of similarity indicates that Cu is present in brain cells in the form of a Cu^+ -thiolate multimetallic cluster and suggests that MT serves as a Cu binding protein.

Metallothionein, a ubiquitously occurring low molecular weight cysteine-rich protein, is involved in numerous physiological processes including metal ion homeostasis, ox-

oxidative stress defense and stimulation of neurite growth [159]. The role of the Cu-MT complex in detoxification of intracellular copper is well established. For instance, one of the therapies received by Wilson's disease patients, whose organisms cannot effectively excrete Cu due to a genetic defect in the ATP7B Cu-transporter, induces metallothionein to facilitate Cu-binding in hepatocytes. While we have not confirmed that Cu accumulations are bound to MT, such an assignment is consistent with current literature. The role of metallothionein in Cu-rich aggregates is further studied with MT knockout mice in Section 5.2

4.2 Nanoprobe XRF analysis of Cu inclusions

To probe the elemental composition of Cu aggregates, it is necessary to have an imaging technique with finer spatial resolution than the size of the particle under analysis. Microprobes (~ 300 nm spot size) technically achieve this resolution for 1 μm diameter particles, but render the particle only a few pixels, making quantitative analysis difficult. Recently, the construction and commissioning of nanoprobe – beamlines with spot sizes smaller than 50 nm – has enabled these particles to be analyzed with adequate spatial resolution. Currently, there are two such beamlines: the Nanoprobe [160] and the Bionanoprobe [161] at the Advanced Photon Source. Both of these beamlines were employed to analyze the ultrastructure of Cu inclusions. It is shown that 1) Cu aggregates are likely spheroids, 2) Cu aggregates rarely co-localize with other metals (e.g. Fe, Zn, Mn), and 3) Cu aggregates consistently co-localize with S at a $[\text{S}]/[\text{Cu}]$ molar ratio consistent with metallothionein being the binding protein.

4.2.1 Experimental description

Male 129S1/SvImJ were purchased from Jackson Labs and sacrificed at 9 weeks old. Mice were chosen for nanoprobe studies as they typically have higher Cu concentrations along the ventricle than rats (Figure 3.3) which makes it easier to find

aggregates to scan. Following the sample preparation protocol detailed in Section 2.5, 5 μm thick coronal sections at Bregma - 0.8 mm were thawed onto 50 nm thick silicon nitride windows that are compatible with both nanoprobe's sample positioning systems. Samples were stored at -80 °C until right before measurements. As both nanoprobe beamlines use vacuum sample environments, samples were dehydrated before loading onto the beamline. Both beamlines were run with an incident energy of 10 keV and had spot sizes of 30 nm (Nanoprobe, 26-ID) or 40 nm (Bionanoprobe, 21-ID).

4.2.2 Results of nanoprobe analysis of Cu inclusions

Often, Cu aggregates imaged with <50 nm resolution appear as spheroid objects (e.g. Figure 4.3D). Since X-ray fluorescence is emitted through the thickness of the entire sample, the recorded maps are two-dimensional projections of the three-dimensional tissue section, and so overlapping spheroids may appear as clusters. Assuming each Cu accumulation is a spheroid-like object, it is possible to deconvolute the separate inclusions. An example is shown (Figure 4.2A) where three spheres are present fitting the intensities of appropriately selected lineplots to a double Gaussian model (Figure 4.2D) confirms that ($R^2 > 0.98$). It is worth noting that there may be some discrepancy in reported numbers from different lines as the accumulations are not perfect spheres and thus the reported values are sensitive to the choice of line. A survey of Cu inclusions in the subventricular zone finds the mean concentration to be 23.60 ± 2.02 $\mu\text{g/g}$ (in a 5 μm section) and the average size of aggregates to be 0.30 ± 0.02 μm (FWHM based on Gaussian fitting.)

Co-localization of the Cu signal with S was prominent in all positions of increased Cu content (Figure 4.2). Linear fits of correlation curves (Figure 4.2B) allowed us to estimate the S/Cu molar ratios in the range of 1.1 to 1.7 (Table 4.1). Increased spatial resolution and sample thicknesses allow a more accurate determination of $[\text{S}]/[\text{Cu}]$. Since fluorescence is recorded from the entire sample thickness, all that can be in-

Figure 4.2. Nanoprobe analysis of Cu inclusions. **A.** A representative Cu hotspot imaged with $30 \times 30 \text{ nm}^2$ pixel size. It is evident that Cu and S co-localize strongly and that accumulations are intracellular. Units are $\mu\text{g}/\text{cm}^2$ in a $5 \mu\text{m}$ thick section. **B.** S/Cu molar ratio only from pixels in the hotspot. Fitting the data to a linear fit yields a S/Cu ratio consistent with assignment of MT(1,2) and/or MT3 (see table 1.) **C.** A close-up on the Cu hotspot featured in A. The three lines correspond to the Cu intensity profiles drawn in D. Yellow dots identify the peak locations when fit to a Gaussian distribution. **D.** Cu intensity profiles corresponding to the green (top), red (middle) and magenta (bottom) lines in C. All three lines are well described by fits to two Gaussians, indicating that Cu hotspots are spheroid-like objects with diameters of around $1 \mu\text{m}$.

Table 4.1.
S/Cu molar ratios at different imaging conditions

Sample Set	Spatial Res- olution	Sample Thickness	[S]/[Cu]
Sprague-Dawley Rats	300 nm	10 μm	1.12 ± 0.07
129S1/SvImJ Mice (MTWT)	300 nm	10 μm	1.18 ± 0.05
129S1/SvImJ Mice (MTWT)	30 nm	5 μm	1.66 ± 0.07
Cu ₁₂ -MT(1,2)	-	-	1.58 - 1.75

ferred is the mean concentration. Since XRF maps of aggregates demonstrate that aggregates are around 1 μm in diameter and very concentrated, the remaining sample lowers the average intensity; there are 9 μm of baseline intensity for microprobe data but only 4 μm for 5 μm thick sections, meaning that [S]/[Cu] from thinner sections should not only be higher, but also more accurate.

While Cu inclusions do not co-localize with other metals, it should be noted that there also exists a separate class of Fe-rich inclusions in the SVZ. To quantitatively determine if Fe and Cu co-localize in individual clusters, images with 30 or 40 nm resolution were thresholded at 17.9 mM for Fe or 31.5 mM for Cu (corresponding to 0.5 and 1.0 $\mu\text{g cm}^{-2}$ in 5 μm thick sections). Particles were then defined by requiring at least one of the surrounding eight pixels to be above the threshold. In other words, if two connected sets of pixels have no connecting pixels they are treated as two separate clusters. To reduce any fluctuations due to noise, only clusters with 5 or more pixels were considered in analysis. For clarity, an example of particle detection is shown in Fig. 4.3B where arrows indicate particles used for analysis. For each cluster, Pearsons correlation coefficient, r , was computed using all pixels in the cluster. A total of $n = 17$ Cu clusters and $n = 22$ Fe clusters were included in analysis from two mice. A summary of correlation coefficients is found in Figure 4.3C. In Cu-rich

clusters, the average correlation was found to be $r = 0.03$ with a median of $r = 0.03$. In Fe rich clusters ($n = 22$ identified clusters), the mean was $r = 0.01$ with a median $r = 0.06$. These data show that Fe and Cu do not co-localize in unstained sections, with rare exception (e.g. Figure 4.3D). The implications of this are discussed in Section 4.3.

Figure 4.3. **A.** XRF map of Fe at 30 nm resolution from an unstained mouse section. In untreated sections, Cu and Fe aggregates tend not co-localize. Scale bar: 500 nm **B.** Demonstration of particle detection on the Fe map in A. Clusters with arrows, which have 5 or more pixels, are used for co-localization analysis. Scale bar: 500 nm. **C.** Co-localization statistics showing Pearsons correlation coefficient for Cu-rich clusters (left) and Fe-rich clusters (right). A boxplot showing the median, 25th percentile, 75th percentile and extremal values is given along with a scatter plot for individual clusters imaged with 30 nm or 40 nm resolution is shown. **D.** Co-localization of Cu, Fe, and P with 40 nm resolution demonstrating a case of Fe-Cu co-localization in the left cluster ($r = 0.49$). Display ranges are given in mM through a 5 μm thick section.

4.3 Iron and Cu separately form the basis for pseudoperoxidase activity in the subventricular zone

Prior to high-resolution elemental imaging, metal-rich inclusions in neurogenic niches were studied by histology, which is not strictly element-specific. Dating back to the early 1970's, it was recognized that many mammals (including humans [162]) possess a subpopulation of glial cells that contain peroxidase activity exhibiting granules [163]. These granules were characterized by their positive staining for Gomori's chrome aluminum hematoxylin, their positive 3-3'-diaminobenzidine (DAB) staining for peroxidase or pseudoperoxidase activity, and orange-red autofluorescence [164]. These granules have come to be known as Gomori-positive (GP) inclusions. Suspecting a porphyrin binding environment causing autofluorescence, researchers in the early 1990's tried to determine the metal basis, if any, for this peroxidase activity. Conflicting reports were published arguing that Cu [165] and Fe [166] give rise to peroxidase activity. While Section 4.2 details that Cu and Fe aggregates exist separately in the SVZ, it did not characterize peroxidase activity in the region. The present experiment aims to determine, by DAB-XRF co-localization, if the Cu inclusions reported by XRF are homologous to the pseudoperoxidase granules described previously. It is shown that both Cu and Fe can produce DAB+ staining.

4.3.1 Experimental Details

A male C57BL/6 mouse (15 weeks old) was sacrificed and sections were taken at Lateral ~ 1.9 mm according to the procedure outlined in Section 2.5. For XRF-DAB co-localization, a 30 μm section was used. Prior to imaging, the section was brought to room temperature and stained for peroxidase or pseudoperoxidase activity. To stain, the sample was incubated in 0.05% DAB in DAB buffer (0.015% H_2O_2 in phosphate buffered saline (PBS)) for 30 minutes. The sample was then incubated for 5 minutes in PBS three times to stop DAB precipitation. To remove excess elements from PBS, the sample was quickly rinsed twice with ultrapure water. The sample

was imaged with an optical microscope both prior to and immediately after X-ray imaging for co-localization with X-ray maps.

X-ray maps were recorded at beamline 2-ID-D at the Advanced Photon Source with a beam size of $250 \times 250 \text{ nm}^2$ and an incident flux of 4×10^9 photons per second at 10 keV. Co-localization was carried out using the landmark correspondence [167] by transforming the optical image to match features on the X-ray maps allowing translation, isotropic scaling, and rotations (i.e. under a similarity transform).

4.3.2 Results of DAB-XRF co-localization

DAB and XRF maps of the same region co-localized are shown in Figure 4.4. It should be noted that the staining process likely modified the metal distribution. DAB+ granules were observed in several situations with respect to metal presence. Some granules are observed in the absence of metal accumulations. Such granules would have either a non-metal or a very dilute metal origin for peroxidase or pseudo peroxidase activity. It is also possible that DAB+ stains that do not co-localize with metal were a result of metals that were washed away during the staining process. More commonly in the SVZ, DAB+ granules do co-localize with Cu, Fe, or Cu and Fe puncta, rendering the metal accumulation a likely source of pseudo peroxidase activity.

This experiment was designed to resolve conflicting claims in the literature that peroxidase activity exhibiting (DAB+) granules derive from Fe complexed with porphyrins [166, 168, 169] while other reports demonstrate that accumulations are Cu rich [165, 170] as measured by energy dispersive spectroscopy (EDS). Similarly, our XRF data with 30-40 nm resolution characterizing untreated mice show Cu-rich aggregates and a separate set of Fe-rich aggregates (Figure 4.3). Analysis of DAB-XRF co-localization compliments nanoprobe analysis in demonstrating that Cu aggregates do not contain Fe and vice-versa. All of the spherical metal accumulations observed,

Figure 4.4. DAB-XRF Co-localization. **A.** Low-magnification micrograph of the DAB-stained section. The arrow points to the rostral corner of the ventricle, which was imaged. Scale bar: 100 μm . **B.** Untransformed optical micrograph of the rostral corner. The dashed line indicates the ventricle wall and the box outlines the area imaged with X-ray microscopy. Scale bar: 10 μm . **C.** Co-localization of Cu, Fe, and optical images showing that DAB+ granules often, but not always, co-localize with metal aggregates. Note that the optical image has been transformed as described in Section 4.3.1. Scale bar: 5 μm .

which was the shape reported as GP inclusions, were primarily composed of Cu and do not co-localize with Fe.

As for the binding protein, Fe aggregates of both porphyrin and non-porphyrin Fe coordination have been proposed. For instance, similar Fe aggregates were recently examined in neurons and were suspected to be siderosomes while hold Fe as ferric hydroxide [171]; ferritin has also been proposed [166]. As both heme and non-heme Fe are capable non-enzymatic peroxidase activity [172,173], the present data is unable to determine a source. With respect to the source of Cu DAB staining, previous work has shown that Cu is present as Cu^+ and as a Cu_xS_y cluster with a $[\text{S}]/[\text{Cu}]$ ratio consistent with that of Cu-metallothionein (Section 3.1, Table 4.1). As metallothionein has an established role in hydrogen peroxide protection [174], it seems plausible that an aggregate containing Cu-metallothionein or a similar Cu-protein cluster could exhibit peroxidase activity.

4.4 X-ray diffraction of aggregates reveals that aggregates have periodic structure

As discussed in Section 1.3, metal-containing protein aggregates have been reported before in the context of disease. While the Cu aggregates under discussion are present in healthy rats and mice, there is a distinct possibility that these aggregates and neurological plaques (e.g. amyloid plaques) are formed by similar mechanisms. The structural hallmark of other metal-containing aggregates is cross- β sheet formation, which exhibits fibrillar diffraction at $4.2 \text{ \AA}$ [175]. The protein tau, which normally is involved with microtubule stabilization, is thought to facilitate cross- β sheet formation following hyperphosphorylation [176]. If Cu aggregates are formed by the same tau-mediated pathway as other cross- β aggregates, then one expects to observe fiber diffraction at $4.2 \text{ \AA}$ from Cu aggregates as well. This experiment was designed to determine if Cu aggregates will exhibit any diffraction; in particular observation of a fiber diffraction ring at $4.2 \text{ \AA}$ would suggest a cross- β folding pattern and one could reasonably expect the mechanisms of other cross- β sheet formation to be applicable to these Cu aggregates. Current data show that Cu aggregates indeed

exhibit Bragg peaks as compared to control tissues, which prominently appear at 3.14 Å. No diffraction rings are observed at 4.2 Å. These data demonstrate that there is some crystalline order in Cu aggregates, which is decidedly different than previously reported fibrillar structures from other metal-containing brain aggregates.

4.4.1 Experimental description

Measurements were made at beamline 2-ID-D at the Advanced Photon Source with an incident energy of 10 keV and a spot size of 250×250 nm². An unfixed, untreated 10 µm thick section from a 15 week old C57BL/6 mouse on thin mylar film was first XRF-scanned. These overview scans allowed the positions of aggregates and nearby control tissue to be recorded. Once the positions were determined, simultaneous XRF and XRD scans were carried out over the aggregate with 60 s exposure times and 300 nm pixel sizes. The diffraction detector, a Mar165 CCD camera, was placed 60 mm from the sample allowing diffraction to be collected over the range $1 \text{ Å} < d < 7 \text{ Å}$. All measurements were made at room temperature in an open air environment.

Diffraction detector calibration was done using CeO₂. A total of 385 exposures from 3 aggregates and 272 exposures from 2 control sections were recorded. To normalize integrated profiles for different exposure numbers, quartic fits were done on the integrated profiles and background normalization was done using those fits.

It should be noted that X-ray induced damage is a concern and in principle could prevent observation of the 4.2 Å ring. Diffraction from Pick bodies (another tau containing cross-β aggregate) has been reported from tissue [177]. These measurements were made with much higher flux ($>100 \times$ total dose delivered) which would rapidly damage the sample; it is reasonable to assume that the conditions reported here do not damage aggregates beyond observing fiber diffraction.

Figure 4.5. XRD from Cu aggregates. **A.** Section of a virtual powder stack for diffraction images recorded from Cu aggregates. The red arrows highlight the peaks observed at 3.14 Å, which is composed of individual Bragg peaks. **B.** Integrated diffraction profiles of aggregates (blue) and control tissue (green). The difference (red) shows several peaks exist in aggregates that do not in normal tissue, the most prominent being at 3.14 Å.

4.4.2 Results of diffraction from Cu-rich aggregates

A virtual powder stack – a sum of recorded images – of diffraction recorded from aggregates is shown in Figure 4.5A. Some of the Bragg peaks that form a peak at 3.14 Å are highlighted with red arrows. This peak is not present in nearby control tissue, as is evident in the integrated powder profiles (Figure 4.5B). It is noted that two peaks are present at 5.76 Å and 5.00 Å in both aggregate scans and control tissue scans (data not shown). Likely these are from the mylar substrate and serve to demonstrate the stability of integration to such high d .

While the present data is insufficient for determining the structure of Cu aggregates, it still serves an important role. The lack of a diffraction ring at 4.2 Å dismisses the notion that Cu aggregates are formed via cross- β sheet formation, like many metal-containing aggregates. Amyloid- β plaques, Pick bodies, Lewy bodies, progres-

sive supranuclear palsy plaques and corticobasal degeneration are all senile plaques that contain cross- β sheets. Tau is expressed in astrocytes and cultured astrocytes have demonstrated the capacity to form tau-positive fibrils in their processes as a response to hyperphosphorylation [178,179]. While tau-induced fibril formation is an appealing mechanism for aggregate formation, the present data suggest another, yet to be determined, aggregation mechanism.

5. The response of Cu in the subventricular zone to aging and insults

One method to determine the role that Cu aggregates play in the brain is to try to alter the Cu level in the subventricular zone through some insult (genetic mutation, dietary modification, exogenous exposure to chemicals, etc.) and correlate altered Cu homeostasis with other changes. The approach has been critical in determining protein function where transgenic animals are routinely used. This chapter is devoted to reporting the array of conditions that were investigated. First, it is shown that Cu in the rat subventricular zone (SVZ) increases with age but not in the cortex or other imaged regions, suggesting an SVZ-unique accumulation mechanism. Secondly, as metallothionein has been proposed to be a feasible candidate for the binding protein (Section 4.1.2), metal concentrations for metallothionein(1,2) knockout (MTKO) mice are characterized. While MTKO mice still exhibit periventricular Cu-rich accumulations, the overall concentration of Cu is lower and there are fewer aggregates in the SVZ demonstrating that metallothionein plays some role in aggregate formation, though cannot be unequivocally assigned as the binding protein. Finally, the cuprizone model for multiple sclerosis – which induces demyelination through dietary Cu chelation – is examined and no changes in metal concentration are reported in either the SVZ or myelin-rich regions are observed suggesting that Cu deficiency may not be responsible for demyelination as previously assumed.

5.1 Aging results in increased Cu in the subventricular zone

One natural question to ask with respect to the role of Cu accumulations is how concentrations vary with age. This information can be elucidating to both the aggregation mechanism as well as any biological function inclusions have in the brain. It is

shown that Cu concentration increases with age specifically in the SVZ as compared to regions surveyed. Furthermore, these increases correlate strongly ($r = 0.99$) with a decrease in neurogenic activity in the subventricular zone.

5.1.1 Experimental description

Male SpragueDawley rats (Harlan, Indianapolis, IN.) were purchased 7-8 weeks old and were housed in a temperature-controlled, 12/12 light/dark room, and allowed free access to pelleted rat chow (Purina rodent chow 5001, 13 ppm Cu content) and distilled, deionized water. This study used in total 12 rats - 3 rats each sacrificed at the four age groups (3 weeks, 12 weeks, 16 weeks, and 36 weeks.) Sections were prepared by the procedure described in (Section 2.5) and at stored at -80 °C until imaged.

XRF imaging was carried out beamline 10.3.2 at the Advanced Light Source [148] and BioCAT (beamline 18-ID) at the Advanced Photon Source [180]. For beamline 10.3.2 data, Cu K_{α} fluorescence was recorded with a Si detector as ROI counts (i.e. not a full spectrum) and normalized for incident intensity and collection time. For BioCAT data, the program MAPS [120] was used to fit the spectrum at each pixel. The results of the Cu quantitation are summarized in Table 5.1. To analyze the Cu concentration in the intracellular compartments, areas containing Cu hot spots were selected by identifying the pixel with maximal Cu content and then taking into consideration all pixels with a Cu content higher or equal to half the maximum. The average Cu concentrations were then obtained using these selected pixels.

To compare the Cu content in the SVZ of rodents of different age median thresholding was employed to isolate Cu hot spots occurring in astrocytes. Median thresholding compares the observed concentration to the local baseline concentration by taking the difference between that pixels concentration and the median of a $30 \times 30 \mu\text{m}^2$ box around that pixel, resulting in a peak concentration map. Analysis of aggregates was carried out using pixels within 90% of the maximum intensity.

For immunolabeling Ki67, frozen (-80 °C) brain sections on microscope slides were thawed, fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) 15 minutes at room temperature (RT), washed three times with PBS, treated for 1 hour at RT sequentially with: 1) blocking/ permeabilization solution: (2% (w/v) bovine serum albumin (BSA) / 0.2% (v/v) Triton X-100 in PBS), 2) rabbit anti-Ki67 at 1:300 dilution in PBS containing 2% BSA at dilutions specified in the reagents listing, 3) Alexa Fluor 488 goat anti-rabbit IgG at 1:1000 dilution in 2% BSA. Three, five minute washes with PBS were performed after treatment with primary and secondary antibodies. Sections were subjected to ProLong Gold antifade reagent before being covered with a coverslip. Slides were sealed and stored at 4 °C. To visualize immunochemical staining a Nikon Eclipse TE2000-U fluorescence inverted microscope was used with simultaneous differential image contrast (DIC) imaging. To quantify Ki67, fluorescing cells were counted as follows: pixels with a fluorescence intensity above a pre-determined threshold (as shown in Fig. 4) which were isolated were considered to be a single cell. To normalize for variations in the ventricle size due to age, the number of fluorescing cells was divided by the length of the ventricle, resulting in cell/ μm units to express number of actively dividing cells. The ventricle length was determined from the differential interference contrast (DIC) micrograph with lengths derived from a camera calibration slide. The ventricles lack of tissue provided sufficient contrast to readily determine the length using the DIC micrograph. Analysis was carried out on three animals at each age (3, 12, 16, and 36 weeks).

5.1.2 Results of aging XRF quantitation

An analysis of the Cu content in the SVZ of animals from 3 weeks to 9 months old showed a dramatic increase in Cu X-ray fluorescence with increased animal age (Figure 5.1). Changes in Cu levels with age were analyzed by measuring two different characteristics: averaged Cu concentration in SVZ (defined as 100 μm layer along the ventricle wall from, Figure 5.1B) and Cu concentration in the aggregates (determined

Figure 5.1. **A.** 3D visualization of normalized Cu K α fluorescence demonstrates an increased copper signal along the lateral ventricle (lv) wall (indicated by the white line) with increased age. Data were obtained from 30 μm thick coronal sections of the brains of 3 weeks, 3 months and 9 months-old rats. Similar distributions were observed in all 9 animals (3 in each age group) used in this study. Experimental details are shown in Appendix A. Insets show visualization of Ki67 proliferation marker. **B.** Circles (blue): Mean Cu concentration in the SVZ as a function of age. Solid line shows fit to monomolecular growth model ($R^2 = 0.944$). Squares (black): Mean Cu concentrations in the cortex of the brain. Triangles (red): Mean Cu concentrations of an entire half-brain coronal section. The solid line in the latter two cases shows a linear regression. It is apparent from this plot that of the regions surveyed Cu accumulation in the brain happens largely in the SVZ throughout aging. **C.** Cu concentrations in the SVZ Cu hotspots determined by median thresholding. The linearity may suggest that Cu accumulation above baseline levels is carried out by a mechanism which does not change its deposition rate with time ($R^2 = 0.56$). **D.** Correlation between the Cu concentrations in the SVZ Cu hotspots and proliferation rate ($r = -0.99$).

by median thresholding, see Section 5.1.1, Figure 5.1C). The observed increase in Cu concentrations with age was fitted to two models: monomolecular growth for tissue level images and linear dependence for 5 μm images where Cu hotspots could be

isolated. For increase of averaged Cu concentration (Figure 5.1B) in SVZ the data were best described ($R^2 = 0.944$) by monomolecular growth: $[Cu] = 80.08(1 - \exp(-(a + 1.377 \text{ wk})/18.03\text{wk}))$ (mM) while increase in Cu concentration in the aggregates was best described by linear fit. Increases in Cu content of the SVZ were compared with data for the cortex (Fig 4B), where essentially no large changes in Cu content were detected. Considering the mean Cu concentration over an entire 30 μm thick coronal half-section also showed a very small increase of Cu concentration with respect to age as compared with growth in the SVZ.

Table 5.1.
Cu quantitation in rats

Method	Sample	Cu Concentration
AAS [181]	Plasma	15-20 μM
AAS [181]	Cerebrospinal Fluid	0.5 μM
XRF	Cu accumulations in cells	$\sim 100\text{-}500 \text{ mM}^\dagger$
XRF	Cu in cells without accumulations	$\sim 130\text{-}300 \mu\text{M}$

† Assumes all Cu is in 1 μm particle

Increased Cu concentration was correlated with a decrease in adult neurogenesis (Figure 5.1D) quantified by IHC for the Ki67 proliferation marker. Strong linear correlations were obtained for both cases of measuring averaged Cu concentration $r = -0.89$ correlation (Pearson) as well as measuring increase in intensity of Cu in aggregates $r = -0.99$ (Figure 5.1D). Design of a specific experiment to assess the effects of Cu-enrichment in type B progenitors on adult neurogenesis is hindered by the currently unknown mechanisms regulating the Cu deposition in these cells. However, it is well known that adult neurogenesis decreases with age which is manifested in the decrease in the self-renewal capacity and the number of SVZ progenitors [182, 183]. The mechanisms responsible for the age-related decline in the function of progenitor cells and other progenitors remain unclear.

The essentially linear increase of Cu concentration in aggregates with age as compared to baseline Cu levels suggests that the mechanism responsible for accumulating Cu is unyielding with age, at least through the ages probed in this study. It has been argued that since natural selection governs metal homeostasis only through reproductive and care-giving ages, neurological mechanisms are not well optimized for aged life; the manifestation of the resulting unoptimized metal concentrations is neurodegenerative disease [137]. Previous studies have established that Cu homeostasis in rats is requisite for reproduction [184] and that trace levels of Cu are necessary to perform functions required for healthy brain development [185]. Migrating neuroblasts lack Cu accumulations (Section 3.2) implying these have been dissolved/consumed or remain with their parent cell during division. Thus, the machinery regulating Cu homeostasis, including Cu stores in the SVZ, in young rodents must be robust to allow sufficient Cu for cell divisions. If that depositing mechanism is never turned off after reproductive ages, however, Cu stores in astrocytes may grow unwieldy. Elevated oxidative stress caused by increased content of redox active metal ions, which has been implicated in Alzheimers disease, Parkinsons disease, and several other neurodegenerative disorders [186], may be capable of preventing proliferation, explaining the observed correlation between peak Cu concentrations and neurogenic rates (Figure 5.1D).

5.2 Metallothionein(I,II) knockout mice exhibit fewer Cu aggregates in the SVZ

Thus far, a wealth of evidence has suggested metallothionein (MT) as a candidate for the binding protein for Cu aggregates. It is the only known protein that is present in GFAP+ cells in the SVZ [187], capable of binding Cu as Cu^+ [151] and aggregating [188], and has a S/Cu ratio consistent with XRF observations (Table 4.1). Furthermore, *in situ* aggregates exhibit almost identical XANES spectra compared to *ex vivo* prepared Cu-MT (Figure 4.1). To determine the role metallothionein plays

in periventricular Cu aggregates, we imaged metallothionein(1,2) knockout (MTKO) mice. (It should be noted there are 3 isoforms of MT in the central nervous system - MT1, MT2, and MT3. A triple knockout rodent has not been reported.) Here we will show XRF-based analysis for age-matched MTWT and MTKO mice. It will be shown that 1) Cu in the SVZ decreases while Zn and Fe are unaffected, 2) this decrease is due in part to the fact that there are fewer inclusions and the present inclusions contain less Cu, 3) remaining inclusions in MTKO mice exhibit consistent XANES spectra compared to MTWT, and 4) in spite of decreased Cu in the SVZ, neurogenesis is unaffected by the mutation.

5.2.1 Experimental description

Male 129S1/SvImJ (MTWT) and 129S7/SvEvBrd-MT1^{tm1Bri}MT2^{tm1Bri} mice – MTKO, an MT(I,II) double knockout mouse – were purchased from Jackson Labs [189]. Mice were sacrificed at either 4 ($n = 3/3$ WT/KO) or 9 ($n = 3/3$) weeks old, as specified in the text. Sections were prepared using the procedure described in Section 2.5. Imaging was carried out at several beamlines for characterization of MTKO mice at several length scales. See Appendix A for imaging details.

XANES for wild type (WT) and MTKO mice were recorded at beamline 20-ID at the Advanced Photon Source. 10 μm thick coronal sections from both 9 week WT and MTKO mice were placed in a Linkam BCS196 cryostage modified to be compatible with X-ray measurements and kept at -100 °C to mitigate X-ray damage. Samples were imaged to determine the location of aggregates from which XANES could be recorded. Scans are calibrated using spectra recorded simultaneously from a reference compound. Damage studies were conducted and no shift in the edge location was detected in more than four scans per spot. The data presented in Figure 5.3 are summed from 114 WT spectra recorded from 28 spots and 28 KO spectra recorded from 14 spots.

For tissue-level resolved images (5 and 20 μm), regions of interest can be differentiated by comparison with a brain atlas and the average metal content of pertinent pixels can be readily determined. For intermediate resolution images (300-500 nm), a static threshold of 10 $\mu\text{g/g}$ was used to isolate hotspots, consistent with previous analysis [190].

5.2.2 Results for metal quantitation of metallothionein knockout mice

For quantitative analysis, four week old MTKO knockout mice were XRF imaged at tissue level (20 μm) resolution and compared with age matched controls. As MT is involved with homeostasis of metals beyond Cu, the concentrations of Cu, Fe, and Zn were characterized. Structures analyzed for differences were the striatum, corpus callosum, cortex, and subventricular zone. XRF did not reveal any statistically significant difference in Cu or other metals for structures examined (Figure 5.2C). With 5 μm resolution, which allows finer isolation of the SVZ, Cu concentrations in the the subventricular zone (Figure 5.2A,D) were significantly lower in MTKO mice. Thus, the overall metal content in the brain is not drastically affected by MT (I,II) knock-out, at least at 4 weeks of age. The SVZ area was also analyzed with 0.5 μm spatial resolution, (Figure 5.2B). The number of Cu aggregates dropped considerably; there were approximately one-third as many Cu aggregates per ventricle length in MTKO mice as compared to wild type mice ($0.152 \mu\text{m}^{-1}$ compared to $0.395 \mu\text{m}^{-1}$). The actual concentration of the Cu in isolated aggregates is also lower (Table 5.2). Finally, both WT and mutant rodents exhibit a $[\text{S}]/[\text{Cu}]$ ratio consistent with previous Cu accumulations (Table 5.2, differences are not statistically significant). It was established earlier that Cu aggregates are localized to astrocytes we checked whether lower number of Cu aggregates in MTKO is due to lower density of astrocytes. Staining of WT and MTKO mice for GFAP we did not detect any differences in the density of astrocytes in the SVZ (data not shown). Finally, quantitation of neurogenesis by

Ki67+ cell density along the SVZ demonstrated no significant difference between WT and MTKO (Table 5.2).

Table 5.2.
Comparison of WT and MTKO mice

	WT	MTKO
Cu aggregate concentration ($\mu\text{g/g}$)*	89.9 ± 5.97	54.9 ± 21.0
S/Cu molar ratio	1.17 ± 0.02	1.10 ± 0.10
Ki67+ cell density (cells/ μm)	0.121 ± 0.014	0.129 ± 0.018
Fzd9+ cell density [†] (cells/ μm)	0.063 ± 0.002	0.050 ± 0.007
* : $p < 0.05$, [†] : $p < 0.10$		

To determine the binding environment for remaining Cu aggregates, XANES spectra were measured from both MTWT and MTKO mice aggregates. The results are shown in Figure 5.3. Spectra from the MTWT and MTKO mice are nearly identical, confirming that remaining clusters are Cu_xS_y multimetallic clusters.

The role of metallothionein in these inclusion bodies remains elusive. Renal studies of Pb exposure found that while WT mice readily produce inclusion bodies, likely as a means to mitigate damage from uptaken Pb, MTKO mice are unable to produce inclusion bodies under the same conditions [188], implicating MT as a necessary component for aggregate formation. Furthermore, transfection of MT into MTKO cells recovered the ability to form aggregates [191]. Just as aggregates form in the kidney in response to lead exposure, it is likely that aggregates form in periventricular stem cells to mitigate oxidative damage from Cu uptake, relying likewise on MT to form stable aggregates.

The Cu rich aggregates are still present in the SVZ, though, even under the MTKO mutation (Figure 5.2). Plausibly this is a result of MT3's presence, which is expressed primarily in the central nervous system and not in the kidney [192,193]. While MT's are capable of aggregating [194,195], it has never been determined which

Figure 5.2. Comparison of MTKO and WT metal concentration and proliferation. **A.** Intensity map of normalized Cu K α XRF with 6 μ m resolution demonstrates the difference in copper concentration along the lateral ventricle wall in control mice (WT) as compared to MTKO mice. White dashed lines indicate the ventricle wall. **B.** Cu XRF allows counting of hotspots along the ventricle wall. WT rodents exhibit 38% more hotspots per micron. Display range is in ppm. **C.** Average metal concentration in various regions of the brain for MTKO (colored) and WT (white) mice. Structures that can readily be identified from XRF maps are the corpus callosum (CC), cortex (CTX), striatum (CPu), and the ventricle. **D.** Comparison of periventricular metal concentrations in MTKO (colored) mice to WT (white) mice. Images of the SVZ with 5 μ m resolution show diminished Cu concentration in the SVZ as a result of the mutation.

Figure 5.3. XANES from tissue of MTWT and MTKO rodents. **A.** Cu K α XRF map from a typical rodent with 10 μ m resolution. The lateral side of the lateral ventricle (LV) is outlined with the dashed line. Arrows indicate spots where XANES scans were recorded. **B.** Summed XANES scans for MTWT (red) and MTKO (blue) rodents recorded directly from tissue.

isoforms facilitate aggregation; the present work suggests that MT3 alone is sufficient for aggregate formation. One consequence of the MTKO mutation is that fewer aggregates formed periventricularly (approximately 1/3 as many per μ m. This is likely a reflection of the fact that MT is not as readily available to promote formation inclusion. Choudhuri et. al. [157] have reported basal relative MT isoform expression in mouse brain to be 1.0, 0.5, and 0.7 for MT1, MT2, and MT3 respectively. In other words MT3 expression constitutes 32% of basal brain MT expression; therefore it is consistent that around 1/3 of the accumulations are observed.

5.3 Mn exposure does not alter Cu concentrations in the SVZ or neurogenesis

A recent article [196] presents a 40% decrease in Cu concentrations of the subventricular zone (SVZ) in rats as measured by atomic absorption spectroscopy (AAS)

attributed to subchronic manganese exposure. Furthermore, that same article reports a statistically significant increase in SVZ and rostral migratory stream (RMS) neurogenesis measured via fluorescence intensity of antibodies for bromodeoxyuridine (BrdU). Interested in the results but skeptical of the methodologies reported, this study was designed to use XRF's superior spatial resolution to validate these findings. In contrast to the reported results [196], XRF analysis data shows a small, significant increase in Cu concentration in the SVZ following Mn treatment but only in areas with increased Cu content. The RMS shows no changes in Cu concentration. Additionally, neurogenic rates were measured by quantifying Ki67, which is expressed in actively dividing cells, by cell counting. This analysis shows that Mn exposure does not affect neurogenic rates in the adult SVZ or RMS.

5.3.1 Experimental description

Animals were prepared in the same treatment groups as those reported by [196]. In particular, male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN) and weighed 250 ± 10 g (mean $\pm$ SD) at the beginning of the experiments. For one week prior to experimentation, the animals acclimated to a temperature-controlled, 12/12-hour light/dark room, and were allowed free access to deionized water and food. Rats in the Mn-treated group received intraperitoneal (i.p.) injections of MnCl_2 dissolved in sterile saline at a dose of 6 mg of Mn/kg once daily for 4 weeks (1 mL/kg). This dose [181, 197] was chosen as it has been shown give rise to blood Mn concentrations that resemble those of exposed to Mn either occupationally or through diet [198], through liver failure [199] and amongst methcathione users [200]. The control group received i.p. injections of sterile saline of the same volume. Sagittal sections from Lateral ~ 1.9 mm were taken by the procedure described in Section 2.5.

XRF imaging was performed at beamline 8-BM of the Advanced Photon Source. Spectra were collected per pixel ($25 \times 25 \mu\text{m}^2$) at an incident energy of 10 keV. The

MAPS program [120] was used to fit the data collected. An XRF image of an AXO thin film reference standard (Axo Dresden GmbH, Dresden, Germany) was taken under the same experimental conditions and geometry which allowed for metal quantification.

For each individual rat sample, line plots were delineated along the SVZ or RMS by the experimenter; the line was thickened by taking each pixel in the user-drawn line as the center of a 7×7 pixel ($150 \times 150 \mu\text{m}^2$) box. As Cu in the SVZ is known to reside mostly in the $100 \mu\text{m}$ nearest to the ventricle [190], selecting only high-concentration pixels serves to spatially isolate the SVZ. To spatially isolate the localized, Cu-rich SVZ or RMS, a Cu concentration threshold of $8.5 \mu\text{g g}^{-1}$ was then applied and the average Cu concentrations determined for both experimental groups for both the SVZ and RMS ($n = 5$ animals per group; see Figure 5.4E,F for an illustration). To determine an appropriate threshold, the lowest concentration that was within one standard deviation of the values obtained when no threshold was applied. By this method, a concentration threshold of $8.5 \mu\text{g g}^{-1}$ was applied to identify Cu accumulations in the desired region. Identified pixels were then used to quantify the average Cu concentration.

To quantify Ki67+ cell density, $10 \mu\text{m}$ thick sections ($n = 4$ per group) were removed from -80°C and allowed to thaw. Sections were then fixed using a 4% paraformaldehyde solution for 15 minutes in followed by three, five-minute washes of phosphate buffered saline (PBS) to remove the fixative. Sections were blocked and permeabilized with 2% bovine serum albumin (BSA) in PBS containing Triton X-100 (0.2%) for 1 hour at room temperature. Primary anti-Ki67 antibody (1:300) diluted in 2% BSA was applied to the sections for 2 hours at room temperature. Following incubation of the primary antibody, sections underwent three, five-minute washes with PBS prior to the application of secondary antibodies. Secondary antibodies (goat anti-mouse Alexa-Fluor 488) diluted 1:1000 in 2% BSA were allowed to incubate for an additional two hours at RT. After three, five-minute washes with PBS, coverslips

were placed over the sections following the application of ProLong Gold antifade reagent.

Ki67 stained sections were imaged at $10\times$ magnification ($0.65\ \mu\text{m}$ / pixel) on an upright Olympus microscope (BX43) equipped with an Olympus DP-72 camera. Puncta of fluorescing cells were blind-counted by hand. Those counting cells were blind to which experimental group (Mn treated or control) an image was from. A site was considered to be a single cell nucleus if there were no strongly fluorescing regions (above a pre-determined threshold) adjacent to it. To normalize for variations in the ventricle or RMS size from potential sectioning artifacts or slight differences in lateral plane location, the number of fluorescing cells was divided by the length of the ventricle or RMS, resulting in units of ($\text{cell}\ \mu\text{m}^{-1}$) to express number of actively dividing cells. $n = 4$ animals in both the control group and treated group were analyzed. Sequential images of the RMS and SVZ were stitched using the ImageJ plugin by [201] with a regression threshold of 0.3 and fusion done by maximum pixel intensity.

5.3.2 Results of Mn exposure on subventricular Cu and neurogenesis

Synchrotron XRF imaging was used to determine the distribution of elements in the rat brain. The XRF imaging showed a strong Cu accumulation both along the SVZ and RMS for all rat samples measured; a typical image is shown in (Figure 5.4C). Since the full X-ray fluorescence spectrum is obtained for each pixel of this image, the distribution of other essential elements are simultaneously recorded. These additional elements can help in identifying the boundaries between neighboring brain regions; for example P and Zn both provide good contrast between the corpus callosum (CC) and the striatum (STR), between which lies the RMS.

Table 5.3 displays the average Cu content of both unthresholded and thresholded pixels ($8.5\ \mu\text{g/g}$) for both the SVZ and RMS in control and treated groups. A small but statistically significant increase in the Cu level in the SVZ of Mn treated animals

Figure 5.4. **A.** Diagram of Lateral 1.90 mm with the approximate location of the displayed XRF scan indicated by the cyan box. **B.** A typical XRF spectrum from an elemental map with elemental K-edges labeled. When an element labels two peaks, these are its K_{α} and K_{β} edges which were both prominent. Peaks that overlap (e.g. K and Ca) are deconvoluted by spectral fitting via the MAPS program. **C.** Cu XRF image with different brain regions labeled. Example of SVZ and RMS selection prior to widening (**D**) and after widening by ± 3 pixels in the four cardinal directions (**E**). Note that widening overestimates the spatial dimensions of both the RMS and SVZ, thereby including neighboring structures (i.e. STR, CC, and/or LV). **F.** Results after applying a concentration threshold of $8.5 \mu\text{g/g}$. Display ranges are in $\mu\text{g/g}$.

was observed in comparison to controls ($p < 0.05$) only after applying a threshold filter. However, the Cu content of the RMS showed no changes ($p = 0.40$). In the same experimental groups, a statistically significant higher Cu level was observed in the SVZ than in RMS ($p < 0.05$).

To quantify neurogenesis in the SVZ and RMS, Ki67+ cells from $n = 4/4$ treated/control rats were counted in $10 \mu\text{m}$ thick sections and quantified by cell counting

Table 5.3.
Mean Cu concentrations following Mn exposure ($\mu\text{g/g}$)

Group	No Threshold	No Threshold	8.5 $\mu\text{g/g}$ Threshold	8.5 $\mu\text{g/g}$ Threshold
	SVZ	RMS	SVZ	RMS
Control	8.58 ± 1.03	7.82 ± 2.02	12.78 ± 0.36	$11.08 \pm 1.06^\ddagger$
Treated	9.82 ± 1.36	8.32 ± 1.94	$13.72 \pm 0.46^*$	$11.84 \pm 1.19^\ddagger$

* : $p < 0.05$ compared to control; † : $p < 0.05$ compared to the SVZ of the same group

‡ : $p < 0.01$ compared to the SVZ of the same group

normalized by length (Figure 5.5). No significant difference was detected in either the SVZ ($p = 0.56$) or the RMS ($p = 0.95$) suggesting that subchronic Mn exposure does not affect adult neurogenesis in these two regions.

Regarding the effect of Mn intoxication on Cu homeostasis in the SVZ and RMS, the present work demonstrate a 7% increase in mean Cu concentration in the SVZ (standard deviations of 3% for both control and treated; $p < 0.05$) and no change in the RMS. This is at odds with [196] which claims that SVZ Cu concentration decreases by 41% while reporting group standard deviations of 26% and 11% respectively for control and treated groups ($p < 0.01$). Animals in both cases were from the same source and treatment group; the only difference was that [196] used AAS and this study uses XRF. A critical difference between the methods is the accuracy with which a given brain region can be identified. As metal distribution in the brain is spatially capricious, [127], resolution is of the utmost importance. From 300 nm resolution XRF maps, it is known that the dense Cu accumulations reside in a single cell layer along the ventricle wall [190] and no reproducible methodology for SVZ extraction by hand dissection has been reported. Experiments isolating the SVZ are of course possible with laser capture microdissection (e.g. [202]) or when including surrounding tissue will not critically affect results, (e.g. [203]). Studies analyzing SVZ metal composition require incredible spatial precision and reproducibility as any accidental inclusion of

Figure 5.5. **A.** Representative Ki67 stain of the SVZ and RMS for a control rat and **B.** Representative Ki67 stain for a Mn treated rat. **C.** High magnification (40 $\times$) view of the RMS in a control rodent. **D.** Quantitation of neurogenesis by Ki67+ cell density in the SVZ and RMS. No significant changes are observed with $n = 4$ animals in each group.

the STR in what is considered the SVZ will lead to artificially and drastically lower Cu concentrations.

The difference in results can be rationalized as a result of the achievable resolution of hand-dissected regions for AAS. It is instructive to compare [196] and the (untreated) rats in [204] in which brains were dissected by hand for AAS. There are two minor differences that must be considered when comparing these studies. First, the rats in [196] received saline injections which should have a minimal effect on brain metal concentration levels. Secondly, rats were 14 weeks old at the time of

sacrifice from [196], placing them near the adult rats group in [204], and certainly concentrations should fall between the adult and old groups. When comparing larger structures like the hippocampal formation (0.633 $\mu\text{g/g}$ vs. 0.81 $\mu\text{g/g}$) and striatum (0.832 $\mu\text{g/g}$ vs. 1.32 $\mu\text{g/g}$) it is evident that hand dissection can provide reproducible concentrations as it has previously in elemental analysis studies. For the SVZ, a small structure, there is almost a 20-fold discrepancy (0.928 $\mu\text{g/g}$ vs. 17.8 $\mu\text{g/g}$). Barring some accidental contamination, this discrepancy is likely a consequence of the difficulties in isolating the SVZ for AAS analysis. In contrast, X-ray fluorescence microscopy (XRF) has spatial resolution which is limited in practice by the measurable, reproducible beam size. Our previous studies using XRF microscopy demonstrate that Cu concentrations are consistently several orders of magnitude higher in Cu-rich accumulations in SVZ than in other brain regions ($\sim 100\text{-}500$ mM). This study shows that Cu-rich accumulations persist along the RMS path and it has been demonstrated that XRF is capable of detecting statistically significant changes in SVZ metal content [190]. From high-resolution XRF maps, Cu is known to reside in a single layer nearly adjacent to the SVZ [190] and a relatively thin layer in the RMS. As a result, dependence in concentrations on the choice of ROI is eliminated by deliberately starting with a very large ROI (± 75 μm) and then applying a concentration threshold which effectively acts to spatially isolate the SVZ or RMS (Figure 5.4F). After applying this threshold a small, but significant ($p < 0.05$) increase in Cu is observed, highlighting the importance of spatial resolution when analyzing metals in such a small, Cu rich region of the brain. XRF analysis on the striatum of this Mn exposure model has previously been reported [205] and no significant changes in any element except Mn were found. For Cu a slight insignificant increase was found in the striatum, but nowhere near the 40% increase reported by [196], which again is likely due to limited dissection resolution for AAS. Similarly, this lab has reported on the HPCf with 7 μm resolution and found no significant changes in other elements under Mn exposure [206].

Quantitation of neurogenesis in the SVZ and RMS showed no statistically significant difference between Mn-treated and control groups (Figure 5.5). This is in disagreement with [196] which reports an increase in neurogenesis by measuring bulk fluorescence of labeled antibodies sensitive to BrdU in the SVZ and RMS rather than by cell counting. Both proliferation markers, Ki67 and BrdU, are localized to the nucleus and thus form discrete, countable puncta under fluorescence. While cell counting of BrdU has been reported since the initial claim of increased neurogenesis [207], it is worth considering that BrdU quantification is susceptible to artifacts of BrdU's uptake into the brain. BrdU is thought to be transported to the brain like thymidine: through a carrier-mediated nucleoside transport system across both the blood-cerebrospinal fluid (BCB) and the blood-brain barrier (BBB) [208–212]. Consequently, comparisons of neurogenesis via quantification of fluorescence intensity of antibodies sensitive to BrdU relies on the assumption that BrdU permeability across the BBB and BCB is unaffected by Mn exposure [213]. While there has not been a study published that examines differential expression of nucleoside transporters at either barrier under Mn exposure, there is a wealth of evidence that the BCB and BBB are affected by Mn exposure. Aside from reports of increased metal transport following Mn insult [35, 214], Santos *et al.* [215] also report differential amino acid concentrations in rat brain tissue following Mn exposure. While the study was unable to distinguish between *de novo* synthesis and transport across the BCB and BBB, there remains a distinct possibility that the barriers have increased permeability after Mn exposure. In studies where barrier integrity is uncompromised (e.g. ischemia), Ki67 and BrdU have been shown to be present in similar ratios between control and insulted groups [213], which is expected as Ki67 is naturally present and any attempts to visualize the protein require steps only after sectioning.

5.4 Cuprizone does not alter Cu concentrations in the subventricular zone or corpus callosum

In 1969, it was first shown that dietary administration of cuprizone – a copper chelator – induces spontaneous demyelination in the central nervous system of mice. Removing cuprizone from the mouse’s diet also leads to remyelination [216]. The cuprizone model reproduces many of the traits of multiple sclerosis (MS) patients: demyelination in the corpus callosum (CC), cerebellum, and cortex as well as axon loss are observed in both MS patients and cuprizone mice [217]. Behaviorally, both MS patients and cuprizone mice show weight loss, diminished motor ability, and have lower activity levels [218,219]. As a result, cuprizone intoxication has become a popular model for studying MS and remyelination. Despite its success, the mechanism by which cuprizone induces demyelination is not known. The current assumption is that Cu chelation leads to Cu deficiency which disrupts cytochrome *c* oxidase activity to the extent that oligodendrocytes cannot produce adequate myelin. In support of that hypothesis, Swiss mice have been reported to have diminished cytochrome *c* oxidase activity following cuprizone administration [220]. The metal concentrations in these demyelinated regions, however, remain unmeasured. Characterization of cuprizone fed mice by X-ray microscopy serves two purposes. First, measurement of Cu concentrations in demyelinated regions will be a direct test of the hypothesis that limited Cu availability causes demyelination. By comparing XRF maps with diffraction-formed myelin maps (Section 2.2) it is possible to correlate the presence of myelin with metals. Secondly, it allows examination of the role that dietary Cu plays in the formation of SVZ Cu aggregates. Data show that while cuprizone fed mice indeed have less myelin in the CC, yet there is no corresponding decrease in Cu. These data suggest the mechanism of demyelination may not be based on Cu availability. In the SVZ, there are no observable changes in metal concentrations.

5.4.1 Experimental details

Eight-week old C57BL/6 mice were fed a diet of standard rodent chow (control group, $n = 4$) or that same chow with 0.2% cuprizone (treatment group, $n = 4$). Mice were maintained on these diets for 5 weeks at which point they were sacrificed and sections were prepared as described in Section 2.5.

X-ray imaging was done at the BioCAT beamline (18-ID) at the Advanced Photon Source. The beam had an incident energy of 12 keV and was focused to a spot size of either $25\text{ }\mu\text{m} \times 4\text{ }\mu\text{m}$ or $10\text{ }\mu\text{m} \times 4\text{ }\mu\text{m}$. X-ray fluorescence was recorded with an energy resolving silicon drift detector. Spectra fitting was done using the MAPS software [120]. The diffraction detector, a Pilatus 1M, was placed 1.5 m behind the sample with a vacuum fly tube between the sample and detector. This allows for data collection in the range $120\text{ }\text{\AA}$ - $11\text{ }\text{\AA}$. To calibrate the diffraction detector geometry, silver behenate was used as a calibrant and calibration was carried out in the Fit2D package [221]. For mapping experiments, ion chambers were placed before the focusing element (I_0) and after the sample (I_1) and their intensities recorded at each pixel for normalization.

Analysis of the cortex, corpus callosum, and striatum are derived from user-defined ROIs from $100\text{ }\mu\text{m}$ images. Metal concentrations in the SVZ are quantified as described in Section 5.3.1 with the threshold being defined as the top 20% of Cu pixel intensities.

Ki67 staining and cell counting was done as described in Section 5.3.1.

5.4.2 XRF and XRD quantification of cuprizone fed mice

XRF maps allowed quantitation of Cu in $30\text{ }\mu\text{m}$ thick sections of brains following cuprizone treatment. Of the cortex, corpus callosum, SVZ, and striatum, no significant differences in Fe, Cu, or Zn concentrations were observed (Table 5.4).

Diffraction and XRF maps were recorded on the same samples on the same pixel grid. In doing so, it is possible to directly correlate myelin concentrations with metal

concentrations. The results of this analysis are shown in Table 5.4. For reference, Figures 5.6A-C show representative myelin and XRF maps in addition to the ROI for the corpus callosum (CC). Quantitation of myelin concentration shows a nearly 30% decrease ($p < 0.05$) for cuprizone fed mice in the CC and cortex after 5 weeks of the diet. XRF analysis in the same region shows that there is no corresponding change in metal concentrations (Table 5.4).

Figure 5.6. Combined XRF and XRD Analysis in the CC. **A-C.** Myelin map, Cu map, and ROI selection for the CC for a representative section. **D.** Quantitation of metals in the CC show no significant changes. **E.** Quantitation of mean myelin concentration in the CC; differences between groups are significant at $p < 0.05$.

Previous quantitation of myelination in the CC following exposure to cuprizone at the 5 week mark have similarly shown a 30% decrease, though other studies have shown much higher demyelination [217,222]. The assumed, though previously unverified, mechanism behind demyelination has always been thought to be deficiency of copper in the corpus callosum which prevents oligodendrocytes from myelin production. Oligodendrocytes are thought to comprise a majority of cells in the CC [223] and so it would be expected that deficiency of Cu in that cell type would manifest itself in XRF measurements. These data suggest a mechanism other oligodendrocytic copper deficiency underlies demyelination. While this mechanism is not yet clear, it should be determined as the cuprizone diet is commonly used to study multiple sclerosis and understanding the demyelination/remyelination mechanism will be useful in developing clinical therapies for MS patients.

Table 5.4.
Quantitation in cuprizone mice

Region	Control Mice	Cuprizone Fed Mice
Fe ($\mu\text{g/g}$)		
Cortex	13.12 ± 2.10	11.43 ± 1.25
Striatum	12.03 ± 0.89	11.42 ± 1.16
Corpus callosum	14.14 ± 4.18	11.00 ± 1.69
SVZ	14.15 ± 3.23	13.27 ± 2.16
Cu ($\mu\text{g/g}$)		
Cortex	3.15 ± 0.09	3.17 ± 0.15
Striatum	3.70 ± 0.83	3.46 ± 0.27
Corpus callosum	3.56 ± 0.56	3.75 ± 0.63
SVZ	14.38 ± 2.10	13.09 ± 1.37
Zn ($\mu\text{g/g}$)		
Cortex	14.10 ± 0.35	13.25 ± 0.54
Striatum	12.97 ± 0.73	12.37 ± 0.76
Corpus callosum	14.11 ± 0.59	13.20 ± 0.90
SVZ	17.03 ± 4.80	12.28 ± 0.62
Relative Myelin Concentration		
Cortex**	1.00 ± 0.06	0.75 ± 0.12
Striatum	1.00 ± 0.17	0.91 ± 0.14
Corpus callosum*	1.00 ± 0.14	0.72 ± 0.16
SVZ	n/a	n/a
Ki67+ Cell Density (cell/ μm)		
SVZ*	0.0539 ± 0.0078	0.0350 ± 0.0101
RMS	0.0541 ± 0.0328	0.0328 ± 0.0128
* : $p < 0.05$; ** : $p < 0.01$ between treatment groups		

6. Conclusions and future directions

This work has extensively used X-ray fluorescence microscopy (XRF) to characterize Cu rich aggregates found in the subventricular zone (SVZ) of rats and mice. In addition, rats, but not mice, have Cu accumulations in the rostral migratory stream and dentate gyrus, which may be related to the fact that the rat hippocampus produces more neurons and new neurons are integrated more readily into neural circuits [131]. Combined XRF and immunohistochemistry enabled determination that aggregates reside in GFAP+ cells and comparison with BrdU labeling indicates that they are in slowly proliferating, likely type-B progenitor cells. Furthermore, Cu aggregates do not co-localize with lysosomes nor are they immunoreactive for ubiquitin, suggesting that the aggregates are not merely awaiting degradation but instead serve some purpose. To elucidate the binding environment XANES spectra were recorded directly from Cu aggregates. These spectra indicate that Cu is bound as Cu^+ as a Cu_xS_y multimetallic cluster. Cu in these aggregates does not co-localize with other metals but consistently co-localizes with S with a molar ratio $[\text{S}]/[\text{Cu}] = 1.66 \pm 0.07$.

To probe the role that these aggregates may play, rodents were subjected to an array of insults and conditions. Cu concentration increases with age specifically in the SVZ, which can be attributed to an increase in the concentration of aggregates. Metallothionein is a candidate for the binding protein given its affinity for binding Cu and its $[\text{S}]/[\text{Cu}]$ ratio. Metallothionein(1,2) knockout mice exhibit fewer aggregates and decreased Cu specifically in the subventricular zone. Aggregates are still present, however, which may be due to the fact that another isoform, metallothionein 3, is present in GFAP+ cells in the subventricular zone. While these data do not permit unequivocal assignment of metallothionein as the binding protein, they do make evident the fact that metallothionein must somehow play a role in the formation of Cu aggregates. Other insults – dietary copper chelation and subchronic Mn exposure –

fail to alter metal concentrations in the SVZ. Neurogenesis as measured by Ki67+ cell density was measured for each of these conditions and insults. With the exception of aging and cuprizone administration, in which the decrease of neurogenesis correlates strongly with increased Cu concentration ($r = 0.99$) though could be a result of other aging effects, no statistically significant changes in neurogenesis were observed in rodents.

It is interesting to speculate on the role that Cu aggregates play in these neurogenic niches. Metallothionein(1,2) knockout (MTKO) mice exhibit both decreased Cu concentrations as well as having fewer stem cells in the SVZ as measured by frizzled 9 (Fzd9) staining. This suggests that stem cell self-renewal is impeded as a result of the inactivation of MT and Cu aggregate formation. One plausible mechanism is disruption of the LIF/STAT3 homeostasis. Metallothionein is known to be induced by STAT1 and STAT3 through a binding site in its promoter region [224]. While the upstream factors governing STAT3 expression are relatively unknown, this hypothesis suggests that there may be some feedback between MT and STAT3 expression which work to maintain appropriate balance for self-renewal of adult stem cells. Even if expression is unchanged, a change in the phosphorylation rate of monomeric STAT3 could account for a lack of self-renewal. Under oxidatively stressed conditions, STAT3 phosphorylation should be increased to correlate to MT upregulation; lower cellular oxidative stress then leads to diminished phosphorylation. This has been directly shown in a handful of systems [225,226]. It has also been shown that oxidative stress promotes dimerization of STAT3 [227]. Both of these would encourage STAT3 translocation to the nucleus for transcription. The present data show lower metal concentrations not only in inclusion bodies but also basally, suggesting that the cell is less stressed oxidatively under insult-free conditions which would slow phosphorylation. From a pluripotency point of view under-phosphorylation of STAT3 would be expected to exhibit the same overall effect as LIF deprivation, namely the inability to self-renew and spontaneous lineage commitment. One possible role for Cu aggregates, then, is that they form to promote self-renewal. However, the unrelenting aggregation

mechanism discussed in Section 5.1 ultimately produces unweidly Cu aggregates in advanced age.

While XRF has served a critical role in discovering and characterizing Cu-rich aggregates, their composition and role cannot be uniquely determined without coupling XRF with standard bench biochemistry. As a first step, the cells which contain Cu aggregates should be cultured. Pastrana *et al.* have reported a method for isolating the major cell types in the SVZ by fluorescence-based cell sorting [228]. Isolated cells of each type could be imaged using XRF to confirm unambiguously the cell type which contains Cu aggregates. Furthermore, these cells could be used to produce aggregates which can be easily used for additional experiments (e.g. mass spectrometry to determine the binding protein). Having established a culture, cells can additionally be subjected to a variety of conditions and easily probed for their response. For example, assays designed to determine phosphorylated versus unphosphorylated STAT3 exist and could be used to test the previous hypothesis with respect to metal concentration. The development of beamline-compatible flow cytometers allows for a direct probe between metal concentrations and protein composition with excellent statistics. The results of these cytology experiments can be used to rationally design *in vivo* experiments and ultimately to help in the design of new clinical therapies.

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APPENDIX

A. Summary of XRF images used in figures

The following is a listing of XRF images presented in this thesis and the imaging conditions they were taken under.

Table A.1.: Details of XRF images

Image	Species	Beamline	Pixel Size (μm^2)	Energy (keV)
2.2	Rat	APS 18-ID	100×100	12.0
2.2	Rat	APS 18-ID	10×10	12.0
3.1	Rat	APS 18-ID	25×25	10.0
3.2	Rat	APS 2-ID-D	0.3×0.3	10.0
3.3A	Rat	APS 8-BM	25×25	10.0
3.3B	Mouse	APS 8-BM	25×25	10.0
3.3C	Rat	APS 8-BM	1.5×1.5	10.0
3.3D	Mouse	APS 18-ID	10×10	10.0
3.4B	Mouse	APS 2-ID-E	0.3×0.3	10.0
3.4D	Rat	APS 2-ID-D	0.3×0.3	10.0
3.5A	Rat	APS 2-ID-D	0.3×0.3	10.0
3.5B	Rat	APS 2-ID-D	0.3×0.3	13.0
3.5C	Rat	APS 2-ID-D	0.3×0.3	13.8
3.6B	Mouse	APS-2-ID-D	0.3×0.3	13.0
3.6D	Rat	APS-2-ID-E	0.3×0.3	13.0
4.1B	Rat	ALS 10.3.2	3×3	11.5
4.1C,D	Rat	ALS 10.3.2	3×3	(varies)
4.2A,C	Mouse	APS 26-ID	0.03×0.03	10.0
4.3A,D	Mouse	APS 21-ID-D	0.04×0.04	10.0
4.4C	Mouse	APS 2-ID-D	0.3×0.3	10.0

4.5 [†]	Mouse	APS 2-ID-D	0.3×0.3	10.0
5.1 [‡]	Rat	ALS 10.3.2	3×3	11.5
5.1 [‡]	Rat	APS 18ID	5×5	10.0
5.2A	Mouse	ALS 10.3.2	3×3	11.5
5.2B	Mouse	APS 2-ID-D	0.5×0.5	10.0
5.2C	Mouse	APS 18-ID-D	25×25	10.0
5.2D	Mouse	APS 18-ID-D	5×5	10.0
5.3A	Mouse	APS 20-ID	10×10	10.0
5.3B	Mouse	APS 20-ID	10×10	(varies)
5.4	Rat	APS 8-BM	25×25	10.0
5.6	Mouse	APS 18-ID	100×100	12.0

[†]: Summed from 1s exposures at these conditions

[‡]: Only 16 week old animals imaged at APS 18-ID

VITA

VITA

Brendan Sullivan received his Bachelor of Science in physics with a minor in computer science from the University of Illinois at Urbana-Champaign in May 2009. During his time there, Brendan worked with Professor Errede to develop a measurement system for near-field acoustic holography and with Nahn Vo, a graduate student in the materials science department at UIUC, to model annealing in Cu-Mo systems following irradiation. Shortly after completing his undergraduate degree, a jazz piece entitled *B.T. Sullivan, Physthythyst* was composed by Joel Caracci.

In August 2009, he moved to West Lafayette, IN to join the Physics and Astronomy program at Purdue University. In October 2011 he joined Professor Yulia Pushkar's group where he learned that it is very fun, and occasionally even productive, to play with synchrotron X-rays. Over the course of four and a half years using synchrotron radiation, Brendan has worked on characterizing the metallome in the brain in addition to providing technical support for time-resolved XAS and XES experiments. Brendan was also a member of Purdue's Physics Graduate Student Association, serving as a Graduate Student Seminar organizer, organization secretary, road adopter, and unofficial cook.