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Metal imaging in neurodegenerative diseases

Megan W. Bourassa^a and Lisa M. Miller^{a,b}

Megan W. Bourassa: megan.bourassa@stonybrook.edu; Lisa M. Miller: lmiller@bnl.gov

^aDepartment of Chemistry, Stony Brook University, Stony Brook, NY, USA. Fax: 631-632-7960; Tel: 631-632-7880

^bPhoton Sciences Directorate, Brookhaven National Laboratory, Upton, NY, USA. Fax: 631-344-3238; Tel: 631-344-2091

Abstract

Metal ions are known to play an important role in many neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and prion diseases. In these diseases, aberrant metal binding or improper regulation of redox active metal ions can induce oxidative stress by producing cytotoxic reactive oxygen species (ROS). Altered metal homeostasis is also frequently seen in the diseased state. As a result, the imaging of metals in intact biological cells and tissues has been very important for understanding the role of metals in neurodegenerative diseases. A wide range of imaging techniques have been utilized, including X-ray fluorescence microscopy (XFM), particle induced X-ray emission (PIXE), energy dispersive X-ray spectroscopy (EDS), laser ablation inductively coupled mass spectrometry (LA-ICP-MS), and secondary ion mass spectrometry (SIMS), all of which allow for the imaging of metals in biological specimens with high spatial resolution and detection sensitivity. These techniques represent unique tools for advancing the understanding of the disease mechanisms and for identifying possible targets for developing treatments. In this review, we will highlight the advances in neurodegenerative disease research facilitated by metal imaging techniques.

Introduction

Neurodegenerative protein-folding diseases are a devastating group of illnesses that involve the misfolding and aggregation of a naturally occurring protein that plays a role in the progressive deterioration of neurons.¹ For a number of years, metals have been implicated in a number of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), prion diseases, and Huntington's disease. However, their precise roles in the disease pathologies have been difficult to elucidate. Redox active metals, specifically copper and iron, are of significant interest because they are capable of forming reactive oxygen species (ROS) through Fenton chemistry.² In this reaction, reduced iron and copper catalyze the production of hydroxyl radicals that damage proteins, DNA, and lipids through oxidative modification. This process results in oxidative stress and eventual cell death. Under normal conditions, these biologically essential metals

are carefully moved through cells using specific chaperone proteins to minimize any aberrant reactions. In the diseased state, it is thought that metal homeostasis is disrupted, resulting in poor control of potentially toxic metal ions. This hypothesis also extends to non-redox active metals such as calcium and zinc,³ which are cytotoxic at high concentrations, which are often associated with neurodegenerative diseases. Therefore, determining the role of metal ions in these diseases has become an important part of understanding these diseases and finding a treatment or a cure.

Imaging of biological metals is an essential tool to gain insight into how metals behave in the diseased state. Using human tissue specimens, animal models, and cell culture models, bio-imaging provides important, spatially-resolved information about how metals behave *in situ*. Traditional methods, such as atomic absorption and inductively coupled plasma mass spectrometry (ICP-MS), provide high detection sensitivity but typically involve bulk measurements using homogenized tissue. As a result, valuable information about the localization of metals on a cellular level is lost. Alternatively, spatial information can be obtained using numerous metal staining methods for cells and tissues, such as the Prussian blue stain for iron. While staining does provide spatially resolved information, it typically lacks the sensitivity for the low concentrations of most biological metals. Staining is also sensitive only to free (unbound) metal ions, and is not easily quantified.⁴ In contrast, bio-imaging techniques allow for the analysis of metal content in tissues or cells with sub-cellular resolution, high detection sensitivity on the order of a few $\mu\text{g g}^{-1}$ or better and methods that are readily quantifiable, providing unique information on cellular mechanisms with respect to neurodegenerative diseases.⁵

This review will focus on the most widely used metal imaging techniques and how they have been applied to neurodegenerative disease research. The advantages of several bio-metal imaging techniques will be discussed, including synchrotron X-ray fluorescence microscopy (XFM), particle induced X-ray emission (PIXE), energy dispersive X-ray spectroscopy (EDS), electron energy loss spectroscopy, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), and secondary ion mass spectrometry (SIMS). In addition, we highlight the impact of these techniques on understanding the role of metal ions in AD, PD, ALS, and prion diseases. Specifically, the high spatial resolution and/or detection sensitivity of these techniques make them ideal for imaging the concentration, distribution, and localization of metal ions in neurodegenerative diseases.

Imaging techniques

In recent years, significant progress has been made to improve the spatial resolution and detection sensitivity of metal imaging techniques. Most techniques offer micron to sub-micron spatial resolution and have a detection limit around 0.1 to 100 $\mu\text{g g}^{-1}$.⁴ These qualities make imaging ideal for studying trace metals at the cellular and subcellular level. Table 1 summarizes the detection limit, spatial resolution, selectivity and analytical depth for each imaging technique discussed below.

The imaging techniques discussed here all follow similar protocols for specimen preparation and data collection. Specimen preparation is fairly straightforward, although the

requirements vary depending on the technique. Cells can often be cultured directly on a thin, trace-metal free substrate. Alternatively, cells can be spun on to a substrate, which is useful for suspension cells or to dilute and disperse cells. Tissues or cells are generally flash-frozen or freeze-dried to preserve the integrity of the specimen. Tissues are typically cryosectioned to approximately 5 to 30 μm thick and mounted on a suitable substrate. Specimens are then placed in front of a focused incident beam (*e.g.* X-rays, protons, or laser). As a result of the beam interacting with the specimen, a particle (*e.g.* photon or ion) is emitted from the specimen and captured by a detector. Generally, the image is created by raster-scanning the specimen through the beam or by moving the beam across the specimen.

Synchrotron X-ray fluorescence microscopy (XFM)

When a beam of X-rays of sufficient energy are focused on to a specimen, the incident X-rays cause the ejection of a core shell electron, leaving a hole. As a result, an outer shell electron will relax to fill the hole by emitting a photon. The energy of the emitted photon is the difference in energies between the two shells and is characteristic of the element from which it originated.⁶ X-ray fluorescence microscopy (XFM) allows for the simultaneous collection of many metal ions ($Z \geq 6$), as long as the incident X-ray beam energy is greater than the binding energy of each element of interest. Recent advances in focusing optics and the high flux of third generation synchrotron sources allow the X-ray beam to be focused down to a few hundred nanometers⁷ (0.1 to 1 μm) and provides sub-cellular resolution.⁸ Using standards of known concentration, XFM data are reliably quantifiable to a detection sensitivity of 0.1 to 1 $\mu\text{g g}^{-1}$. The high energy of the X-ray beam also allows for a large analytical depth greater than 100 μm . However, tissue specimens are normally much thinner to avoid analyzing multiple layers of cells. Specimens are mounted on thin trace-metal free substrates, such as silicon nitride or Ultralene.⁸

X-ray absorption spectroscopy (XAS) can often be performed using the same experimental set up as XFM. This technique scans the energy of the incident monochromatic X-ray beam across the absorption edge of an element of interest. X-ray absorption near edge structure (XANES) extends from a few eV before the absorption edge to about 150 eV above the edge and provides information about the oxidation state of the element. Extended X-ray absorption fine structure (EXAFS), from ~150 to 800 eV above the absorption edge, shows the interference between the emitted photoelectron and the neighbouring atoms, which create a backscattering effect. The backscattering can provide structural information within approximately a 5 $\text{\AA}$ radius of the atom of interest.⁹ XAS has been useful in studying active sites of metalloproteins including speciation and ligand binding.¹⁰

Particle induced X-ray emission (PIXE)

Particle Induced X-ray Emission (PIXE) operates under the same X-ray photon emission phenomenon as XFM, but the incident beam is composed of particles (generally protons) rather than X-rays. The particle beam encounters a metal ion, causing the ejection of an inner shell electron. An outer shell electron then relaxes, emitting a photon, which is then detected. The energy of the emitted photon is dependent upon the originating atom. The proton beam generally has an energy range between 2–4 MeV¹¹ and allows for the simultaneous collection of any element (all Z). This technique has a spatial resolution of 0.2

to 2 μm and a detection limit of 1 to 10 $\mu\text{g g}^{-1}$. The analytical depth of PIXE is between 10 and 100 μm . Similar to XFM, specimens need to be thin, dry, and mounted on a metal-free substrate.

PIXE is easily coupled with other techniques such as Rutherford backscattering (RBS) and scanning transmission ion microscopy (STIM). Together these three techniques provide very complementary data. RBS measures the energy scattered off of the particles after the interaction with the specimen. RBS is especially sensitive to lighter atoms such as carbon, nitrogen, and oxygen, which are not easily measured by PIXE, and can be used to determine specimen thickness and density.¹¹ STIM measures the energy loss of the incident particles after hitting the specimen and can also measure the specimen thickness. With appropriate standards, PIXE data is readily quantifiable.

Energy dispersive X-ray spectroscopy (EDS)

Energy Dispersive X-ray Spectroscopy (EDS), also referred to as Electron Photon Micro Analysis (EPMA), utilizes an electron beam to analyze a specimen. Similar to PIXE and XFM, the electron beam causes the ejection of an inner shell electron, resulting in the emission of an X-ray photon from the atom as an outer electron relaxes to fill the inner shell hole. Each element has a characteristic X-ray photon equal to the energy difference between the two shells, and a full energy dispersive spectrum is generally collected at each point for simultaneous analysis of multiple elements ($Z \geq 6$). EDS allows for high spatial resolution between 30 and 40 nm¹² and a detection limit between 100 and 1000 $\mu\text{g g}^{-1}$. EDS is quantifiable using standards. The analytical depth is between 0.1 and 1 μm . EDS is often coupled with transmission electron microscopy (TEM) or scanning electron microscopy (SEM) to provide additional morphological information about a specimen.

Thin sections of specimens are often placed on TEM grids. These grids are usually made of nickel, copper or beryllium with conductive carbon film substrates.¹³ Similar to the other imaging techniques, biological specimen preparation for EDS usually involves flash freezing and freeze-drying to preserve the sample.

Electron energy loss spectroscopy (EELS)

Electron energy loss spectroscopy (EELS) utilizes an electron beam of known kinetic energy and measures the energy lost by the electron through inelastic scattering with the specimen. This energy loss is characteristic of the element that scattered the electron. Information about chemical bonds and the electronic structure of the material can be obtained.¹⁴ EELS is especially useful for imaging low-Z elements like carbon, but can be used with elements as large as the 3d metals (through zinc). EELS has a spatial resolution around 0.001 μm and single atoms, such as iron and calcium, have been detected in biological specimens.¹⁵ The detection sensitivity is approximately 1000 $\mu\text{g g}^{-1}$ and an analytical depth of 50 nm makes EELS a surface sensitive technique. EELS is often used in conjunction with EDS, TEM or SEM.

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

Laser Ablation-ICP-MS (LA-ICP-MS) utilizes a focused laser beam to ionize part of a specimen. The laser-ablated specimen is sent through an ICP, which atomizes and ionizes the specimen.¹⁶ The ions are then sent through an MS and separated by their mass-to-charge ratio. In this way, isotopic information can be attained as well as elemental analysis. Since the laser ablates only one area at a time, this technique is spatially resolved. Current lasers can be focused to about 5 to 150 μm on the specimen. LA-ICP-MS has a detection limit of approximately $0.01 \mu\text{g g}^{-1}$. LA-ICP-MS is destructive to the specimen because a laser leaves behind a small pit the size of the analytical volume ($\sim 200 \mu\text{m}$ deep). This technique can be difficult to quantify due to a lack of standard reference materials so homemade matrix-matched standards are required for quantification.¹⁷ Cryosections of biological tissues are also recommended for LA-ICP-MS, usually around 20–30 μm thick. Sections can be placed on TEM grids for electron microscope analysis prior to LA-ICP-MS. Using thicker specimens, three-dimensional images have been created by repeatedly raster scanning a specimen. As the ablated region is roughly the same depth at each point, multiple images can be created and stacked to form a three-dimensional image.¹⁸

Secondary ion mass spectrometry (SIMS)

Secondary Ion Mass Spectrometry (SIMS) imaging measures the secondary ions that are removed from the specimen as a result of the collision with a primary ion beam. The secondary ions are separated using their mass to charge ratio measured with a quadrupole or time-of-flight mass analyzer.¹⁹ The primary ion beam (*e.g.* cesium or molecular oxygen cations) used depends on the element or molecule of interest. For the detection of most metals, an oxygen ion beam is often used because it favors the formation of positive ions. Similar to LA-ICP-MS, this method is also destructive to the specimen.²⁰ The latest instruments provide $\sim 50 \text{ nm}$ lateral resolution.¹² SIMS operates in one of two modes, static or dynamic. Static SIMS uses a lower intensity primary beam, stays very close to the specimen's surface, and generates larger fragments such as intact molecules. Dynamic SIMS requires a higher intensity beam, and has a detection limit around $0.01 \mu\text{g g}^{-1}$. This technique penetrates deeper into the specimen (0.1 μm) and produces smaller fragments, making it ideal for elemental analysis.⁴ SIMS experiments can be more difficult to perform because the technique requires specimens to withstand ultra-high vacuum in the analysis chamber.²¹

Applications

Alzheimer's disease

AD is the most common age-related neurodegenerative disease, affecting approximately 2% of the population in industrialized countries.²² Clinically, AD is characterized by memory loss and diminished cognitive functioning. Pathologically, amyloid plaques and neurofibrillary tangles (NFT) are found in the AD brain.²³ The amyloid plaques consist primarily of aggregated amyloid- β ($\text{A}\beta$) peptide, which is produced by the cleavage of the amyloid precursor protein (APP). The functions of both $\text{A}\beta$ and APP are unclear, but there is evidence that APP may regulate neuronal survival, neurite outgrowth, synaptic plasticity, and cell adhesion.²⁴ NFTs are formed from paired helical filaments of hyperphosphorylated

tau, a microtubule-associated protein, and are found in other neurodegenerative diseases such as frontotemporal dementia.²⁵

It is becoming clear that AD is a complex disease that involves other factors related to protein aggregation.²⁶ For example, amyloid accumulations have been found in cognitively normal specimens and some AD patients have no amyloid deposits.²⁷ Additionally, a number of amyloid-independent mechanisms have been linked to AD. For example, the familial AD mutations in APP and PS1 can lead to defective autophagy and lysosomal proteolysis,^{26,28,29} and there is strong evidence that reduced cerebral blood flow, as in chronic cerebral hypoperfusion, is strongly related to an increased propensity for developing AD.³⁰ The complexity of AD has made the etiology of the disease very difficult to elucidate.

Metals have been implicated in AD for more than half a century, when large iron deposits were observed in AD brains using a Prussian blue iron stain.³¹ Since then, the role of metal ions such as iron, copper, zinc, and aluminium, has been studied extensively in AD. In recent years, numerous metal imaging studies have been performed on both human tissue and AD mouse models to determine the distribution of metal ions in neurological tissue and understand if/how metal ion homeostasis is disrupted during the course of the disease. However, the precise role of metal ions in the disease pathology is still unclear.

Metals and A β plaques

Human A β plaques have been shown to contain a high concentration of metal ions, and copper and zinc have been co-purified with A β plaques.³² Several metal imaging studies have also demonstrated relatively high concentrations of metals inside the plaques compared to surrounding tissue. Specifically, Lovell and coworkers used PIXE to show that the rim and core of human senile plaques contained elevated iron, copper, and zinc.³³ Similar findings were reported more recently using XFM, where iron, copper, and zinc were elevated in the plaques compared to the surrounding tissue in human AD specimens by 177%, 466%, and 339%, respectively.^{34,35}

The A β peptide contains binding sites for both copper and zinc *via* histidine residues, which may account for their presence in the plaque.³⁶ Copper is able to bind A β ^{1–42} with attomolar affinity and can induce the precipitation of A β in mildly acidic conditions.³⁶ The AD brain is slightly acidic so the presence of copper could provide a mechanism for plaque formation. Copper also interacts with A β to form oligomers, which are thought to be the most neurotoxic form of the protein.³⁷ Copper-binding to A β involves a redox process that reduces copper while creating damaging ROS, such as hydrogen peroxide, that are toxic to neurons.^{32,38} Interestingly, A β was not toxic to cells in culture without copper (II) present.³² Zinc can also bind to A β but with lower (micromolar) affinity and is capable of inducing A β aggregation at physiological pH.^{39,40}

In vitro experiments have shown that the strict removal of metal ions from buffer solutions can inhibit A β aggregation.⁴¹ This indicates that very low metal ion concentrations can induce aggregation; thus, there could be enough free copper and zinc from synaptic transmissions to form A β aggregates.^{42,43} Metal chelators have been suggested as a potential therapeutic option to inhibit A β aggregation. The use of clioquinol, a copper and zinc

chelator, has reduced the A β plaque burden in Tg2576 mice by 49%.⁴⁴ Treatment of the same AD mouse model with tetrathiomolybdate, a copper complexing agent, reduced copper levels in the brain and A β plaque burden when used preventatively, but not when used as a treatment.⁴⁵ This suggests that early treatment may be critical to reducing plaque burden through chelation.

Unlike copper and zinc, the iron in the plaques does not appear to directly interact with A β .⁴⁶ The presence of iron in human A β plaques was originally identified through immunostaining as ferritin, the primary iron storage protein in the brain.⁴⁷ More recently, XAS was used to identify biogenic magnetite and/or maghemite in the plaque cores of human specimens, suggesting an abnormal biomineralization process is occurring within the plaque or surrounding tissue.^{48,49} The size distribution of the magnetite cores implies the formation of a ferritin precursor, implicating a malfunction in ferritin. Magnetite contains a mixture of iron (II) and iron (III) so it also capable of inducing oxidative stress.

Mouse models of AD provide several advantages over human studies in the control of genetics and for time course studies to determine the order of pathological events. One such model is the PSAPP mouse, which develops amyloid pathology as well as learning and memory impairments by 6 months of age, but does not show signs of neurodegeneration.⁵⁰ Using XFM and normalized to the protein density in the plaque, these mice did not exhibit an elevation in copper or iron throughout the course of the disease, and only a slight increase in zinc was found at the late stages of the disease (Fig. 1).^{35,51} In contrast, the CRND-8 mouse, which develops amyloid pathology and memory deficits at a younger age (*i.e.* by 3 months), as well as neuronal and axonal degeneration,^{52,53} showed a 2- to 3-fold increase in the amount of iron, copper, and zinc in the plaques at 6 months of age, as demonstrated by PIXE coupled with STM and RBS.⁵⁴ Similar findings were observed with LA-ICP-MS analysis of plaques from the TASTPM mouse that develops plaques at 4 months of age.⁵⁵

In all of the mouse models, the relatively small amount of metal in the plaques compared to human plaques is very intriguing. However, since the life span of a mouse is only ~2 years, mouse plaques are typically “younger” than human plaques, suggesting that, as plaques “age”, they accumulate metal ions. In other words, metal ions do not likely cause plaques to form; rather, metal-binding occurs over time, where there is a time lapse between plaque formation and metal accumulation, such that older plaques contain more metal.

The metal content in the plaques is also correlated with the degree of neurodegeneration, where humans exhibit high metal concentration in the plaques and high neurodegeneration, whereas the mouse models show very little metal uptake and neuron death. The differences between the human plaques and those in the mouse models could demonstrate that an imbalance of metal homeostasis is a source of metal ions that accumulate in the plaques. Alternatively, the “aged” metallated plaques could provide a source of toxic metal ions.

In addition to the brain, the retina has also been shown to have several abnormalities in early-AD patients⁵⁶ and contain A β plaques.⁵⁷ The retina can be readily imaged non-invasively with high resolution and sensitivity using optical coherence tomography (OCT), which has been done successfully with live AD mice (APP_{SW}E/PS1_{E9}).⁵⁸ It was recently

shown that functionalized iron oxide microparticles can be used as contrast agents to improve the quality of OCT images.⁵⁹ Thus, it is possible that these iron oxide microparticles could be used to identify A β plaques in the retina, and could provide a valuable tool for early AD diagnosis.

Metals and NFTs

Tau stabilizes neuronal microtubules, aids in neuronal development and plasticity,⁶⁰ and is the primary component in NFTs.²⁵ Alpha-helical segments of hyperphosphorylated tau are known to form paired helical filaments (PHFs) that can become NFTs. NFTs have also been associated with metal abnormalities including a 10-fold increase in iron and a 6-fold increase in copper. Smaller increases in zinc have been found in ubiquitin positive areas associated with human NFTs.^{61–63}

Tau contains multiple microtubule binding domains where the second and third binding domains have an affinity for copper.^{60,64} With the addition of copper, both domains are capable of converting to an alpha-helical conformation, indicating copper can enhance the formation of the NFTs.⁶⁰ Iron (III) has also been found to induce aggregation of hyperphosphorylated tau and can form PHFs, similar to copper.⁶⁵ Additionally, the treatment of iron (III)-containing tau aggregates with reducing agents *in vitro* releases iron (II) and re-solubilizes the aggregates. The release of iron (II) could further add to the oxidative stress seen in AD through the creation of ROS.

Aluminium and Alzheimer's disease

In addition to physiologically-essential metals such as iron, copper, and zinc, several studies have implicated aluminium in AD, as it has also been found to associate with A β plaques and NFTs. Aluminium was first linked to AD in 1965 when alum-treated rabbits were found to have NFTs similar to those seen in human AD patients.^{66,67} Pearl and Brody later showed that neurons with NFTs contained aluminium,⁶⁸ which was directly bound to the NFTs.⁶⁹ A few other studies have also found aluminium in the NFTs and senile plaques from human tissues using EDS.^{49,70–72} However, the majority of published studies did not find aluminium in the NFTs or plaques and suggested that experimental flaws accounted for the presence of aluminium in previous studies.^{73–75} For example, a large increase in aluminium was found in NFTs and healthy tissue fixed with osmium tetroxide, a common tissue fixative, but not in unfixed flash frozen specimens. Thus specimen preparation may have contaminated the tissues with aluminium.⁷⁶ Other studies have indicated that the aluminium may be a result of dust on the specimen.^{49,71} Therefore, careful specimen preparation and handling are important factors in obtaining accurate metal data.

Despite its possible presence in plaques and NFTs, aluminium is not believed to be the etiologic origin of AD.^{77,78} Aluminium is likely brought into the body through food and the environment⁷⁹ and enters through the weakened blood brain barrier of AD patients,⁸⁰ where it is able to interact with the plaques and NFTs.

Metal homeostasis

In addition to metal ions accumulating in plaques and NFTs, metal ion dysregulation has been observed in AD. Using PIXE, elevated zinc was found in the amygdala, hippocampus,⁸¹ and neuropils³³ of human AD brains. The increased zinc is potentially attributed to the zinc enriched neurons (ZEN) found in these regions, which maintain a pool of zinc for synaptic release and contain zinc transporter proteins. It is hypothesized that zinc released from the neurons can react with A β to facilitate aggregation⁸² as zinc can induce the precipitation of A β .^{40,83} The precipitation of A β is considered neuroprotective because it eliminates the more toxic oligomeric form.⁸⁴ However, the sequestration of zinc by A β can prevent it from reaching its normal physiological targets, such as NMDA receptors,⁸⁵ where zinc acts as an inhibitor.⁸⁶ Without sufficient zinc, excitotoxicity can occur. Additionally, the reuptake of zinc is energy dependent, and in AD, it has been suggested that mitochondrial energy production failure results in increased free zinc, which can also interact with A β .⁸⁵

Iron homeostasis is also altered in AD. Elevated iron was found in several human studies in the motor cortex,⁸⁷ hippocampus,⁸⁸ basal ganglia, putamen,⁸⁹ cerebellum and cortex.⁹⁰ In the PSAPP mouse model, an increase in iron was also found in the cortex and hippocampus.³⁵ Fig. 2 shows the distribution of iron, as well as zinc and copper, from the hippocampus of PSAPP mice using XFM. This increase was observed early in the disease – coincident with the onset of plaque formation. Ferritin, the primary iron storage protein in the brain, is often thought to be involved in the changes in iron homeostasis in the AD brain.⁸⁷ Increased iron was found in the brains of APP/V7171 mice, and determined to be primarily iron (III) and likely complexed with ferritin, based on XAS analysis.⁹¹ Using TEM and nano-SIMS, ferritin has been observed in the coronal region of human AD plaques, which is associated with the non-A β component of the plaques where other proteins are located.⁹² Ferritin was also co-localized with hemosiderin in the sulfur-rich dystrophic neurites and in the glial cells. The presence of ferritin and hemosiderin indicate impaired iron clearance. It is possible that the ferritin, which normally stores redox inactive iron (III), may become dysfunctional in the AD brain and bind iron (II), leading to oxidative stress reactions. Additionally, hemosiderin binds iron (II) only loosely, potentially resulting in further oxidative damage.

While the reason for altered iron homeostasis is unclear, the excess iron can have major consequences that may impact the progression of AD. First, the increased presence of iron can lead to the production of ROS. Oxidative damage from ROS is known to kill neurons, but may also extend to other cellular components. For example, evidence of lipid peroxidation has also been observed in the PSAPP mouse model of AD.⁹³ Second, an increase in iron results in increased APP expression and provides the potential for more A β production.⁹⁴ Iron is also associated with furin, which modulates α -secretase and β -secretase activity. When iron levels are high, the furin activity is decreased, encouraging the use of β -secretase, which produces the amyloidogenic A β .^{95,96}

The increase in iron also demonstrates its role as a potential biomarker for early diagnosis through clinical imaging because amyloid pathology is thought to develop well before the onset of symptoms. This would be key to treating AD because pre-clinical stages are thought

to occur years before the onset of symptoms.⁹⁷ Early identification would allow treatments to begin before significant neuronal death has occurred, when they are more likely to be effective.³⁵

Parkinson's disease

PD is the second most common neurodegenerative disease. Symptoms of PD include tremors, rigidity, and difficulty walking. Advanced stages of the disease bring on cognitive and behavioral deficits such as dementia. PD is characterized by a loss of dopaminergic neurons, specifically in the substantia nigra (SN), resulting in decreased production of the neurotransmitter dopamine. Dopamine is the precursor to neuromelanin, the pigment that gives the SN its dark color.⁹⁸ PD patients also develop cytoplasmic Lewy bodies, which contain aggregates of α -synuclein, a protein whose normal physiological and pathological roles are unknown.⁹⁹ As PD progresses, the effectiveness of levodopa, the most common treatment for the disease, diminishes. The drug also has numerous severe side effects including psychosis and dyskinesia,¹⁰⁰ which makes new treatment options highly desirable.

Metals have been shown to interact with both α -synuclein and neuromelanin. Studies have shown that α -synuclein has at least three copper (II) binding sites.¹⁰¹ Upon binding copper, the aggregation propensity of α -synuclein increases.¹⁰² In contrast, neuromelanin has the ability to bind iron with high affinity, thus suggesting that it plays a neuroprotective role by limiting free radical damage.¹⁰³ Therefore, both copper and iron homeostasis may be affected in PD, making them the primary elements of interest in metal imaging studies.⁶¹

PD is similar to manganism, which results from toxic exposure to manganese, and is most commonly found in miners, smelters, and welders who have high exposure to manganese. While the symptoms of manganism and PD are very similar, the globus pallidus (GP) is the most affected brain region in manganism rather than the SN.¹⁰⁴ Due to the disease similarities, manganese is also thought to play a role in PD and is frequently used in PD models.

Metals in the substantia nigra and cortex

In PD, the metal concentration and distribution in the brain have been shown to be altered. Specifically, XFM has been used to show an increase in iron, copper, and zinc in human SN neurons,¹⁰⁵ where iron shows the largest increase in the midbrain SN. Additional XFM experiments showed that iron was also elevated in the laminae that defines the putamen and globus pallidus of human PD specimens.¹⁰⁶ Interestingly, the iron content was independent of the density of remaining dopaminergic neurons.¹⁰⁷ This lack of correlation indicates that iron elevation is an intrinsic change in PD, but is not related to disease severity. Moreover, XAS studies have shown that iron (III) is the predominant iron species in the SN neurons of both PD specimens and healthy controls.¹⁰⁸ Copper speciation also identified predominately as copper (II) in single nerve cells in the SN, though no differences in copper speciation were found between human PD and control specimens.¹⁰⁹ The oxidized forms of iron (III) and copper (II) are less prone to redox damage *via* ROS formation

The cause of elevated iron content is unclear, but iron metabolism, including iron transport, uptake, and storage, is believed to be disrupted in PD.^{110,111} The increased iron is attributed to iron transport proteins, such as transferrin, ferritin, and divalent metal transporter-1 (DMT-1).¹¹¹ DMT-1 expression is increased after exposure to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP), causing iron accumulation and a loss of dopaminergic neurons.¹¹² Transferrin is increased in the dopaminergic neurons of the SN in PD rat and monkey models.¹¹³ Oxidative modification of transferrin by rotenone, a neurotoxin linked to PD, is known to release ferrous iron. Increased ferritin has also been associated with PD in the basal ganglia, even at very early stages of the disease.¹¹⁴ Thus, all of these events involving iron proteins result in additional iron in the PD brain and increase the potential for oxidative stress.

Metals and α -synuclein

α -Synuclein is a known metal-binding protein. Metals added to α -synuclein monomers *in vitro* have decreased the aggregation time from 2 weeks with no metal to 1 day with iron, aluminium, cobalt, and copper.¹⁰² While α -synuclein is capable of binding several metals, it has an exceptionally high affinity for copper. *In vitro* studies have shown that α -synuclein contains 2 high affinity copper binding sites (~ 0.1 – $50 \mu\text{M}$) and at least one additional copper binding site with a lower affinity (400 – $500 \mu\text{M}$).¹⁰¹ As an intrinsically disordered protein, it has been hypothesized that the long-range interactions are disrupted upon metal binding, which subsequently destabilizes the protein and induces aggregation.^{101,115,116} In the cytoplasm, α -synuclein is known to be unstructured but maintains an α -helical conformation in the plasma membrane.¹¹⁷ With 24 negatively charged residues at neutral pH,¹¹⁸ metal binding is hypothesized to neutralize these charges;¹⁰² though, the effect of copper binding to α -synuclein and subsequent oligomerization remains unclear.¹¹⁹

Metals and neuromelanin

Neuromelanin is also capable of binding metals, specifically iron.¹²⁰ Approximately 10–20% of the iron found in a healthy SN is bound to neuromelanin.¹²¹ The binding sites in neuromelanin include catechol, carboxylic acid, and benzothiazine functional groups.¹²² PIXE imaging of the SN of human specimens showed iron colocalized with neuromelanin in the neurons but there was no difference in iron concentration between PD and healthy control subjects (see Fig. 3).^{61,123} Moreover, XFM studies of neuromelanin granules revealed that those from glial cells and those released from dead cells had the largest amounts of iron.¹²⁴ Less iron was found in the neuromelanin of surviving neurons. Neuromelanin appears to bind primarily iron (III) and is suggested to be an iron storage compound in dopaminergic neurons.^{103,125} In the neuromelanin granules, the spectroscopy showed a shift from iron (II) towards iron (III) as the cells deteriorated,¹²⁶ suggesting the iron changed in the neurodegenerative process, possibly through the production of ROS.

Neuromelanin has been shown to reduce hydroxyl radicals, a product of the Fenton reaction and oxidative stress.¹²⁷ As a result, neuromelanin is generally considered neuroprotective. However, neuromelanin is potentially toxic when metal concentrations are too high^{111,128} because the iron binding sites contain different affinities. At low concentrations of iron, the high affinity binding sites are used first. However, the weaker binding sites are used in cases

of high iron concentrations, as in PD, resulting in looser iron binding and the potential for the production of more hydroxyl radicals.¹⁰³

PD animal models

Several animal models of PD exist, but rather than transgenic mice, most of the models involve exposure to toxins such as 6-hydroxydopamine (6OHDA),¹⁸ MPTP,¹²⁹ or manganese,¹³⁰ all of which cause Parkinsonian-like symptoms.

6OHDA is a neurotoxin that results in the loss of catecholaminergic neurons when injected into the SN, producing PD symptoms (reviewed in ref. 131). The toxin is brought into the neurons by dopamine and noradrenergic transporter molecules. The mechanism of toxicity is thought to involve the production of hydroxyl radicals in the presence of iron. In mice sacrificed 21 days after an injection with 6-OHDA, LA-ICP-MS results showed a 20% increase in iron in the SN on the side of the 6OHDA injection.¹⁸ This indicates a disease-dependent accumulation of iron in the SN, which could lead to increased ROS production.

MPTP is a highly lipophilic neurotoxin that readily crosses the blood brain barrier (reviewed in ref. 131). MPTP is converted to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP) by monoamine oxidase in non-dopaminergic cells. MPDP oxidizes to form a 1-methyl-4-phenylpyridinium cation (MPP+) and is released by the cells. MPP+ has high affinity for dopamine, noradrenaline, and serotonin transporters, which result in the uptake of MPP+ into the dopaminergic neurons where it can impair mitochondrial function. Unilateral MPTP-lesioning caused iron content to increase 5-fold in the SN of old *versus* young lesioned monkeys, as determined by PIXE analysis.¹³⁰ Comparing the lesioned to unlesioned (control) side of the SN, no difference in the young monkeys was observed but a 10% iron increase in the lesioned side of the older monkeys was found, indicating an age-related susceptibility to the neurotoxin. In the older monkeys, the iron was associated with intracellular ferrous iron-rich deposits in the SN. In addition, a direct correlation was found between the iron concentration and the level of iron (II), which can more readily promote free radicals and other ROS than iron (III).¹³²

Using LA-ICP-MS, mice injected with MPTP showed a significant decrease in copper concentrations in the periventricular zone and fascia dentate.¹²⁹ MPTP affects the cellular energy metabolism by depletion of adenosine triphosphate (ATP). Thus soon after MPTP exposure, it is plausible that copper uptake is limited. However, 28 days after the last MPTP injection, copper levels were elevated by 40% in these regions, which is thought to be a compensatory mechanism associated with recovery from exposure to the toxin, as shown in Fig. 4. Soon after exposure to MPTP, iron concentrations were increased in the interpeduncular nucleus by about 40%, but decreased slightly in the SN. Iron levels remained increased in the interpeduncular nucleus 28 days after MPTP injection but recovered to normal levels in the SN. The results of this MPTP study are opposite those of the previously mentioned 6-OHDA studies, where increases in iron were seen up to 21 days after the injection with 6-OHDA. While the MPTP model represents a wider range of PD symptoms than 6-OHDA, the effects of the MPTP are more reversible, which may account for the differences in results between the two models.¹³³

Rats exposed to moderate levels of manganese showed no ultrastructural damage in the neurons and glial cells of the basal ganglia.¹³⁴ However, the mitochondria of astrocytes were found to have a 700% increase in manganese compared to astrocyte mitochondria from control mice as measured by EELS and electron spectroscopy imaging. The mitochondria in the neurons also showed a 200% increase in manganese compared to control neurons. Mitochondria are known to accumulate manganese and may play a role in manganese homeostasis. The extreme manganese levels in the astrocyte mitochondria compared to the other cell types may be associated with glutamine synthetase, a manganoprotein that catalyzes the conversion of glutamic acid to glutamine. Glutamine synthetase, produced by the astrocytes, is the primary manganese component in the brain, comprising an estimated 80% of total brain manganese,¹³⁵ and glutamine synthase levels are affected by with manganese exposure.¹³⁶ The nucleolus and heterochromatin of the neurons and the astrocytes also showed increases in manganese. This increase may indicate that manganese is interacting with and potentially damaging the inactive heterochromatin and the active nucleolus.¹³⁴

The toxicity-induced PD models produce acute symptoms, but to model the progressive neurodegeneration seen in PD, transgenic mouse models are more commonly used. One such model uses an iron regulatory protein (IRP1^{+/-} IRP2^{-/-}) knockout mouse.¹³⁷ This model better mimics the chronic and progressive neurodegeneration and demonstrates the importance of iron homeostasis in the disease. In the knockout mice, EELS data showed a 10-fold decrease in ferritin in the axonal space compared to wild-type, despite an overall increase in total ferritin in cerebellar neurons. This reduction in ferritin from the degenerating neurons indicates there is a problem with iron trafficking even at early stages of neurodegeneration.¹³⁷

PD cell culture models

Cell culture models of PD generally use PC12 cells, which synthesize and store dopamine in large neurovesicles. When exposed to nerve growth factor, the cells terminally differentiate and develop neurite-like processes, similar to the dopaminergic neurons affected in PD.¹³⁸ Based on XFM data, cells exposed to subtoxic and toxic levels of manganese sequestered the manganese in the Golgi.¹⁰⁴ However, cells exposed to toxic levels of manganese also contained manganese surrounding the nucleus. This indicates that manganese toxicity may occur when the Golgi becomes overloaded. Cells that were exposed to both manganese and brefeldin A, a compound that disintegrates the Golgi apparatus, showed a redistribution of manganese around the cell, specifically in the cytoplasm and the nucleus.¹⁰⁴ The large concentrations of manganese in the Golgi indicate the Golgi's vital role in manganese detoxification in the cell, but when the Golgi is compromised manganese is allowed to spread throughout the cell. There is recent evidence that proteins involved in preventing PD, such as PARK9¹³⁹ and parkin¹⁴⁰ play protective role against manganese toxicity. It is hypothesized that PARK9 and parkin may also be neuroprotective by maintaining the integrity of the Golgi apparatus.¹⁰⁴

Significant improvements in the spatial resolution of PIXE and XFM have allowed for imaging at subcellular resolution. PIXE imaging of PC12 cells exposed to excess iron

showed elevated iron in all cellular components but most notably in the distal ends.¹³⁸ Copper and zinc also accumulated abnormally in the neurite-like outgrowths, as demonstrated by XFM.¹⁴¹ In a similar experiment, results found increased iron in the dopamine neurovesicles.¹⁴² The presence of iron-dopamine complexes suggests that dopamine may play a neuroprotective role in iron chelation. After exposure to α -methyltyrosine (ATM), an inhibitor of dopamine synthesis, iron concentrations diminished in the cells, especially the dopamine neurovesicles. It has been suggested that mutations in α -synuclein, found in familial PD, can reduce the number of dopamine neurovesicles and result in a redistribution of the iron-dopamine complexes.¹⁴³ Without the proper sequestration of iron in the dopamine neurovesicles, the highly oxidizing iron-dopamine complex could lead to increased oxidative stress and a decrease in the dopamine neurovesicles. Additionally, the progressive loss of dopaminergic neurons in PD could lead to diminished control of the increased iron, which can result in additional oxidative damage.

Amyotrophic lateral sclerosis

ALS is the most common motor neuron disease, with a lifetime risk of 1 in 2000.¹⁴⁴ It is characterized by motor neuron degeneration that leads to muscle weakness followed by progressive paralysis and eventually respiratory failure. ALS is generally fatal within 3 to 5 years from the onset of symptoms. Interestingly, the age of onset is highly correlated to the rate of disease progression and survival time after the onset of symptoms. Juvenile ALS (onset before 25 years of age), as well as late onset (after the age of 65) ALS are very rare but, generally lead to very rapid progression and significantly shorter survival times than average patients (between 45 and 65 years old).^{145,146} The disease primarily affects the spinal cord and, at later stages, the brain stem. Approximately 90% of ALS cases are sporadic (sALS), with no known cause, but the remaining 10% have a genetic link. ALS cases with a known genetic cause are termed familial (fALS).

fALS with copper-zinc superoxide dismutase

One quarter of fALS cases are associated with mutations in the *SOD1* gene, which codes for the protein copper-zinc superoxide dismutase (SOD1). Any one of over 160 known mutations in this protein can cause the disease.¹⁴⁷ SOD1 acts as an essential antioxidant metalloenzyme, which binds one zinc atom for stability and one copper atom to neutralize superoxide radicals. In ALS, it is hypothesized that mutant forms of the protein undergo a “toxic gain of function,” in which the protein becomes a pro-oxidant, rather than an antioxidant. The gain of function is associated with aberrant metal binding or activity.¹⁴⁴ Therefore, the effects of metals ions in ALS, especially copper and zinc have been studied extensively.¹⁴⁴ The misfolded mutant SOD1 forms aggregates in the spinal cord motor neurons of patients. Its aggregation is thought to be neuroprotective as it sequesters the toxic soluble protein.

SOD1 mutations have a large variation in biophysical and chemical properties. Mutations are classified as either wild type-like (WTL) or metal-binding-region (MBR) mutations. The WTL mutations, such as G37R and G93A, are capable of binding copper and zinc and often maintain similar activity levels to the wild-type (WT) protein. In contrast, MBR mutations,

such as H46R/H48Q, are unable to bind metal or do so with reduced affinity. As a result, the activity levels of MBR mutations are significantly reduced in comparison to WT SOD1.

Mouse models of SOD1 fALS provide excellent insight in to the disease. Mice expressing mutated human SOD1 develop progressive paralysis and SOD1 aggregates in the spinal cord motor neurons. HPLC-ICP-MS studies performed on SOD1 transgenic mice revealed that insoluble fractions of SOD1 were not enriched in metal, while the soluble fractions of WT and WTL mutations were highly metallated. Given the high stability of the WTL mutations, G37R and G93A, it is unlikely that the mature metallated proteins will aggregate. Therefore, the lack of metal in the insoluble fraction supports the hypothesis that nascent and immature proteins are more prone to aggregation.¹⁴⁸ In an XFM study of the same mouse models of ALS, an increase in copper in the gray matter, where the SOD1 is expressed, was found in the WT and WTL mutant specimens, relative to non-transgenic (NTG) mice.¹⁴⁸ However, this change was not seen in H46R/H48Q, a mutant that does not bind metal. A disease-specific increase in zinc was also found in the white matter of all mutants. The cause of these changes is unclear, but taken together they represent an alteration of metal homeostasis in the diseased state. Based on XFM images of zinc content, motor neuron loss was evident; Fig. 5 shows large motor neurons in the WT mouse that are absent in the symptomatic mice expressing G85R SOD1.

sALS

The etiology of sALS is unknown, although it is thought to be a multifactorial disease that may include a genetic susceptibility and environmental triggers.^{149,150} Altered metal homeostasis is also thought to play a role in sALS. Using XFM, elevated zinc concentrations were found in the gray matter compared to the white matter in the spinal cords of sALS specimens.¹⁵¹ Despite the lack of redox activity in zinc, there is still the potential that changes in zinc homeostasis can result in oxidative stress by zinc ions interacting with mitochondrial metabolism.¹⁵² Zinc is also a component of glutamate excitotoxicity, which is found in patients with sALS (and fALS).¹⁵³ Here, elevated zinc increases glutamate release from neurons in a toxic manner.

Pigmented creatine deposits have also been found in the brain stem and spinal cord of some sALS patients; however, their origin is unknown. XFM studies of human sALS specimens did not reveal any metal (iron, copper or zinc) in the deposits.¹⁵⁴ The lack of metal indicates that they did not originate from the blood stream or from neuromelanin, as these sources would likely contain metal. Supplements of creatine, an organic acid involved in ATP production, have been correlated with increased survival time in ALS mouse models.¹⁵⁵ Unfortunately, human trials with creatine supplements have had mixed results.

Prion diseases

Prion diseases are fatal neurodegenerative diseases in which the normal prion protein PrP^C undergoes a conformational change to the pathogenic form PrP^{Sc}. Some studies have suggested that this process involves copper binding, where PrP^C undergoes a conformational change and increases in α -helical content while decreasing in β -sheet structure.¹⁵⁶ Indeed,

PrP^C contains four high-affinity copper binding sites that consist of an octarepeat sequence (PHGGGWGQ) and a fifth site with lower affinity.^{157–159}

The precise role of PrP^C is unknown, but it has been suggested to be a copper transport protein because it can bind multiple copper ions through its histidine and glycine residues in the octarepeat. The copper-PrP^C complex can be endocytosed by a cell *via* clathrin coated pits, indicating PrP^C's potential role in copper uptake after presynaptic depolarization.¹⁶⁰ The affinity for the copper binding sites is highly pH dependent and copper binding becomes unstable below a pH of 6.5. The pH dependence provides evidence for PrP^C as a copper transporter that detects copper (II) in the extracellular matrix and then releases the copper in the lower pH of the endosomes.¹⁵⁹ There is also evidence that PrP^C plays numerous roles in other cellular processes.¹⁶¹ For example, PrP^C can protect cultured cells against apoptosis induced by Bax, a pro-apoptotic protein.^{162,163} Primary neurons from Prnp^{0/0} mice were shown to be more susceptible to damage from ROS than neurons from WT mice, suggesting PrP^C's role as an antioxidant.^{164,165} Additionally, PrP^C is likely to be involved in transmembrane signalling for neuronal survival and neurite outgrowth.^{166–169}

There is also significant evidence that PrP^C can mitigate oxidative stress but it is unclear whether this effect is due to the protein sequestering redox active copper or by directly acting as an antioxidant.¹⁷⁰ Recent XAS studies have shown that zinc (II) binds to PrP^C with lower affinity than copper, and induces copper (II) to bind in a redox-inactive coordination geometry, reducing the level of ROS produced.¹⁷¹ In prion diseases, oxidative stress is especially evident in the endoplasmic reticulum (ER), where calreticulin, a calcium-binding protein that resides in the ER, was recently found to inhibit aggregation of PrP^C (23–98) *in vitro*.¹⁷² Modulating aggregation could increase oxidative damage as it may promote the smaller oligomeric form, which is generally considered more toxic.

Given the pathogenic nature of prion diseases, they have been primarily studied using animal models. In a XFM study using WT PrP mice, prion gene knock-out (*Prnp*^{-/-}) mice, and overexpressing PrP^C (Tga20) mice, the WT mice showed more metal in the brain than the knock-out mice and less metal than the overexpressing mice, indicating that the prion protein influences metal ion homeostasis.¹⁶⁰ Moreover, areas of increased or decreased metal content corresponded with regions of higher or low PrP^C expression, respectively. The presence of copper binding sites in PrP^C provides further support regarding the increased presence of copper in regions of the brain where PrP^C is over expressed.¹⁶⁰ Specifically, the knockout mice showed lower copper in the periventricular regions, lower iron in the thalamus, hippocampus and olfactory bulbs, and reduced zinc in the hippocampus. However, in the posterior region of the lateral ventricles, iron and zinc were increased.¹⁶⁰ The overexpressing PrP^C (Tga20) mice had increased iron and copper in most regions of the brain compared to WT mice. Copper was especially high in the periventricular region, while iron was highest in the medial septal nucleus and the septal hypothalamic nucleus. However, the Tga20 mice had decreased iron, copper, and zinc in the posterior hypothalamus. The correlation between altered metal concentrations and PrP^C expression demonstrates that PrP^C may be involved in the regulation and distribution of metals in the central nervous system.

Clinical imaging

Due to altered metal homeostasis in a wide range of neurodegenerative diseases, clinical imaging of patients has the potential to play an important role in early diagnosis, monitoring disease progression, and the development of drug therapies. In the clinical setting, the most common metal imaging tool is magnetic resonance imaging (MRI) using iron due to its magnetic properties. With recent advances in high field-strength MRI, iron is now readily detectable at physiological concentrations.¹⁷³ Copper and manganese also possess magnetic properties but are present in lower concentrations in the brain so imaging of these metals is extremely difficult.¹⁷⁴ While MRI techniques possess diminished spatial resolution (~2.4 mm at 3T) compared to traditional metal imaging techniques discussed here,¹⁷⁵ MRI provides sufficient resolution to detect regional differences and has the significant advantage of imaging living patients rather than being restricted post-mortem analysis.¹⁷⁴

Using MRI, regional increases in iron have been seen in the brains of patients with several neurological diseases, including AD and PD.¹⁷⁶ In AD, increased iron was found in the basal ganglia, specifically the caudate nucleus and putamen as determined using field dependent R2 increase (FDRI).^{177,178} The increase in iron indicates that it may be a biomarker for the disease.^{89,178} In patients reporting memory loss, House *et al.*¹⁷⁹ found increased iron in the right temporal cortex but decreased iron in the left internal capsule compared to control subjects who reported no memory loss. They also found decreased iron levels in the frontal and temporal white matter compared to controls.¹⁷⁹ The reduction of iron in the white matter is thought to reflect a loss of myelin in these regions. Thus, these disease-dependent changes could be important biomarkers for disease diagnosis, which is especially valuable for AD, where diagnosis today can only be confirmed by post-mortem analysis.

MRI has also been used to visualize the iron in brains of patients with PD. Increased iron has been found in the SN pars compacta, dentate nucleus, subthalamic nucleus, and basal ganglia.¹⁸⁰ Several studies have been able to correlate an increase in iron in the SN with an increase in disease severity.^{175,181–183} In a study using susceptibility-weighted imaging (SWI), no difference was seen in iron content in the SN between early-onset (~40 years old) and later-onset (>60 years old) PD patients, but an increase in iron was found in all PD cases compared to age- and sex- matched controls.¹⁸³ These studies further demonstrate the potential for disturbances in iron metabolism in PD and could prove to be a powerful biomarker in the diagnosis and treatment of the disease using MRI.

Positron emission tomography (PET) is another clinical imaging technique that shows significant promise for patients with neurodegenerative diseases.¹⁸⁴ Advances in PET imaging have aided in the early diagnosis of PD through the use of radiotracers such as ¹⁸F-FDOPA, where studies have shown a reduced uptake of the radiotracer in the brain of PD patients.¹⁸⁵ In addition, single photon emission computed tomography (SPECT), which is similar to PET but utilizes gamma ray emission, has helped to discriminate between idiopathic PD and other parkinsonian syndromes, which is important for assessing the prognosis and associated therapy.^{186,187} Oxidative stress has also been imaged in PD patients using a copper-62 labelled diacetyl-bis(N⁴-methylthiosemicarbazone)

radiopharmaceutical, which accumulates in regions of the brain where there are excessive electrons relative to O₂ that reduce the copper (II) to copper (I).¹⁸⁸

In AD, significant advances have been made by quantifying amyloid burden with PET imaging using the Pittsburgh Compound B (PiB), a carbon-11 labelled thioflavin-T analogue.^{189,190} Unfortunately a number of cognitively normal subjects show a significant amyloid burden, which complicates the interpretation.¹⁹¹ However, a newer approaches exploit the changes copper homeostasis using a copper-64-labelled *bis*(thiosemicarbazonato)copper complex, which has a longer retention time in animal models of AD compared to control animals.¹⁹² The longer half life of copper-64 radiolabels (~12.7 h) compared to carbon-11 (20.3 min) also allows for longer synthesis times and regional distribution.¹⁹² Fluorescent copper sensors have also been developed to visualize labile copper pools in animal models of Wilson disease, a disease associated with copper overload, and in the future could be applied to neurodegenerative diseases like AD and ALS.¹⁹³ With the aid of copper radiolabels and the ability to detect copper pools, alterations in copper homeostasis can be utilized to improve our understandings of the mechanisms of copper homeostasis and provide better diagnostics for neurodegenerative diseases.

Conclusions

Imaging of biological metals can aid in understanding the mechanisms of neurodegenerative diseases, as well as in their diagnosis and treatment. Imaging will continue to grow as a powerful tool for uncovering the role of metal ions in neurodegenerative diseases. Improvements in spatial resolution and detection sensitivity will be critical for providing more detailed information about the localization of metal ions, particularly on the sub-cellular level. This could be especially useful in understanding the effects of neurodegenerative diseases on the nucleus and mitochondria, which are both heavily impacted in the diseased state. Clinical imaging techniques will also prove to be invaluable in confirming the diagnosis of these diseases through the use of biomarkers, such as iron. In addition, clinical imaging offers the unique ability to monitor disease progression in patients and to determine the effects of novel drug treatments. Metal imaging techniques will continue to help expand the knowledge base of neurodegenerative diseases and provide a systematic approach to deconstructing complex mechanisms occurring in the body and may ultimately lead to a cure for neurodegenerative diseases.

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Biographies

Megan W. Bourassa

Megan Bourassa received her B.S. in Chemistry from Pacific Lutheran University in Tacoma, Washington. She recently completed her Ph.D. in Chemistry at Stony Brook University, performing her research at the National Synchrotron Light Source at Brookhaven National Laboratory in the group of Dr. Lisa Miller. Her research involves the role of metal ions in amyotrophic lateral sclerosis (ALS) and Alzheimer's disease using both X-ray fluorescence and infrared microscopy.

Lisa M. Miller

Lisa Miller is a senior biophysical chemist and Associate Division Director for Spectroscopy and Imaging at the National Synchrotron Light Source at Brookhaven National Laboratory. She is also an adjunct Associate Professor in the Departments of Chemistry and Biomedical Engineering at Stony Brook University. She obtained her Ph.D. in Biophysics from the Albert Einstein College of Medicine in 1995. In 1999, Lisa became a staff scientist at BNL and received tenure in 2006. Her research involves developing and using x-ray and infrared imaging techniques to study diseases such as osteoarthritis, osteoporosis, and Alzheimer's disease.

Fig. 1. XFM reveals the metal content within the plaques and surrounding tissue of PSAPP mice, a model of AD. (A) Thioflavin S-stained PSAPP mouse brain tissue. XFM microprobe images of (B) zinc, (C) copper, (D) iron, and (E) calcium distribution in the same tissue. (F) XFM microprobe spectra from the center of a plaque (cyan) and from the surrounding normal tissue (red). All scale bars are 5 µm. (From Leskovjan *et al.*⁵¹).

Fig. 2.

XFM analysis of iron, copper, and zinc from the PSAPP mouse hippocampus. Spatial distribution of iron, copper, and zinc in the hippocampus of PSAPP and CNT mice measured using XFM. (A) H&E stained hippocampal brain section from a PSAPP mouse. XFM images of (B) iron, (C) zinc, and (D) copper in a serial tissue section. Units are mM. Scale bar=300 μ m. (E) Hierarchical cluster analysis (HCA) was used to create unsupervised regions of interest (ROIs) based on iron, copper, and zinc content in order to compare metal content in separate regions of the hippocampus. On average, four clusters were required to separate the images into histological ROIs where the dendritic layer is gray, the PCL is green, CA3 is blue, and the hilus is magenta. These areas corresponded to distinct anatomical regions of the hippocampus defining four distinct regions of the hippocampus. (F) Average XFM spectra from each ROI (from Leskovjan *et al.*³⁵).

Fig. 3.

Visible light image (left) and PIXE elemental maps for phosphorus, sulfur, iron, calcium, copper and nickel from two neuromelanin containing dopaminergic neurons, from a control specimen (top) and from a PD specimen (bottom). The iron image shows that there is no difference in concentration between the control and the PD specimen. Reprinted from *Nucl. Instrum. Methods Phys. Res., Sect. B*, **260**, Reinert *et al.*, High resolution quantitative element mapping of neuromelanin-containing neurons, pages 227–230, copyright 2007, with permission from Elsevier.¹²³

Fig. 4.

LA-ICP-MS metal images of copper, iron, zinc and manganese representative of each group (control, 2 h, 7 d, and 28 d after the last of five daily MPTP injections). Sections are on a posterior level crossing the substantia nigra, the interpeduncular nucleus, and the hippocampus. Reprinted from *J. Am. Soc. Mass Spectrom.*, **21**, Matusch *et al.*, Cerebral bioimaging of Cu, Fe, Zn, and Mn in the MPTP mouse model of Parkinson's disease using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), pages 161–171, copyright 2010, with permission from Elsevier.¹²⁹

Fig. 5.

XFM images of iron, copper, and zinc from a portion of the ventral horn of a healthy WT mouse (top) and a symptomatic G85R mouse (bottom). Several motor neurons can be seen in the zinc image from the WT mice, while no intact motor neurons are visible in the G85R mice. The G85R mice also show an increase in iron and zinc in the gray matter.

Table 1

Primary analytical imaging techniques for analysis of biological metals with the respective detection limit, spatial resolution, element selectivity, and analytical depth for each technique. Adapted from Lobinski *et al.*⁴

Analytical imaging technique	Detection limit ($\mu\text{g g}^{-1}$)	Spatial resolution (μm)	Selectivity	Analytical depth (μm)	References
XFM	0.1–1	0.1–1	Multielemental (Z 10)	>100	AD ^{31,34,35,62,73,91} PD ^{104–106,125,142,143} ALS ^{149,152,155} Prion diseases ¹⁶¹
μ -XAS	100	0.1–1	Chemical species	>100	AD ^{48,62,91} PD ^{108,109,133} Prion diseases ¹⁷²
PIXE	1–10	0.2–2	Multielemental (all Z)	10–100	AD ^{34,54,74,81} PD ^{123,124,131,139}
EDS (EPMA)	100–1000	0.03–0.04	Multielemental (Z 6)	0.1–1	AD ^{49,70–72} PD ¹⁰⁷
EELS	1000	0.001	Multielemental (Z 6) chemical species	<0.05	AD ⁴⁹ PD ^{135,137}
LA-ICP MS	0.01	5–150	Multielemental and isotopic	200	AD ⁵⁵ PD ^{18,130}
SIMS (dynamic mode)	0.1	0.05	Multielemental and isotopic	0.1	AD ⁹²

XFM: X-ray Fluorescence Microscopy; μ -XAS: micro-X-ray Absorption Spectroscopy; PIXE: Particle Induced X-ray Emission; EDS: Energy Dispersive X-ray Spectroscopy; EPMA: Electron Probe Microanalysis; EELS: Electron Energy Loss Spectroscopy; LA-ICP MS: Laser Ablation Inductively Coupled Plasma Mass Spectrometry; SIMS: Secondary Ion Mass Spectrometry; AD: Alzheimer's Disease; PD: Parkinson's Disease; ALS: Amyotrophic Lateral Sclerosis.