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A review of the "metallome" within neurons and glia, as revealed by elemental mapping of brain tissue

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ABSTRACT

It is now well established that transition metals, such as Iron (Fe), Copper (Cu), and Zinc (Zn) are necessary for healthy brain function. Although Fe, Cu, and Zn are essential to the brain, imbalances in the amount, distribution, or chemical form ("metallome") of these metals is linked to the pathology of numerous brain diseases or disorders. Despite the known importance of metal ions for both brain health and disease, the metallome that exists within specific types of brain cells is yet to be fully characterised. The aim of this mini-review is to present an overview of the current knowledge of the metallome found within specific brain cells (oligodendrocytes, astrocytes, microglia, and neurons), as revealed by direct elemental mapping techniques. It is hoped this review will foster continued research using direct elemental mapping techniques to fully characterise the brain cell metallome.

Introduction

In general terms, brain cells can be classified into two types, neurons and glia. The defining anatomical features of neurons are dendrites and axons, and their defining physiological characteristic is the ability to generate an action potential. Neurons can be further divided into a range of sub-classes, such as pyramidal neurons, motor neurons, Purkinje neurons, and interneurons to name a few. Even within these classes, further sub-division is possible based on the nature of synaptic connections and the neurotransmitters used [1,2]. In contrast to neurons, glial cells do not generate an action potential. Although glia contain many fibre like "processes", they do not contain dendrites or axons. As with neurons, the glia lineage can be further broken down into cell types, such as astrocytes, microglia, and oligodendrocytes. In general, astrocytes provide critical metabolic support for brain neurons, microglia are the resident immune cells of the brain, and oligodendrocytes produce the lipid rich myelin sheath that insulates axons [3–5]. Indeed, as is the case with neurons and interneurons, further sub-division and classifications of astrocytes, microglia, and oligodendrocytes is possible, but is beyond the scope of this review.

In recent years there have been major advances in our understanding of the metabolome, genome, and proteome of different types of brain cells, and how this relates to healthy brain function, or brain malfunction (e.g., during disease or after injury). Despite the large advances in metabolomics, proteomics, and genomics, our understanding of brain metallomics remains largely incomplete. The cell metallome refers to the complement of different chemical forms of metal ions that exist within a cell. It is critical for the field of neuroscience to continue to further identify, characterise, and understand the metallome within different brain cells, as there is substantive evidence linking balanced metal homoeostasis to healthy brain function [6–11]. Further, metal dyshomeostasis is strongly implicated in neurodegeneration and cognitive decline in multiple diseases (e.g., Alzheimer's disease, [6,12–19] Parkinson's disease [20–23], amyotrophic lateral sclerosis [24,25], multiple sclerosis [26–28]), neurological disorders (e.g., epilepsy [29–31]), or following brain injuries (e.g., traumatic injury [32–34] and stroke [35–41]).

As a consequence of coordination chemistry, the transition metal ions (Fe, Cu, and Zn) are able to deliver a diverse range of chemical function to support cell biology. A detailed description of the wide range of cell processes supported by metal ions is beyond the scope of this review, but many excellent reviews already exist on the topic [6,8–11, 42–45]. Detailed knowledge of the differences in the metallome between cell types and cell sub-types can shed vital information on the inherent functioning of brain cells, or identify potential pathways or conditions that may render certain cells vulnerable to damage or impaired function

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[12,13,46,47]. As an example, the classical histochemical method for detecting brain Fe, the Perl's stain, has repeatedly shown oligodendrocytes are enriched in Fe [48,49]. Knowledge of the abundance of histochemically detectable Fe within oligodendrocytes has been linked to the high metabolic turnover required for myelin synthesis [4,50]. This knowledge of Fe metallomics within oligodendrocytes has directed research to investigate the possible susceptibility of oligodendrocytes to oxidative stress during disease or injury, along an axis of Fe-mediated free-radical production [51]. Another prominent example of where knowledge of brain metallomics has provided valuable insight into disease pathology, was the discovery of metal enrichment within amyloid-β plaques during Alzheimer's disease [52–59]. Fe, Cu, and Zn enrichment has been observed in amyloid-β plaques, in human and pre-clinical animal tissues [52–59], which subsequently drove studies to reveal that metal ions can catalyse fibril formation, possibly driving or accelerating plaque formation and associated pathologies (e.g. oxidative stress) [16,60–65].

The above examples are just two cases where knowledge of the brain metallome provides vital insight into pathways of disease. Despite the clear importance of studying the metallome of brain cells, progress is not always easy, largely due to the difficulty in imaging and quantifying the different chemical forms of metal ions found in different brain cells. Although a range of cells can be grown in cell culture, it is well established that brain cells *in vitro* can be metabolically and physiologically different to brain cells *in vivo* [66]. It is therefore, imperative to study the metallome of different brain cell types and sub-types in conditions as close as possible to that found *in vivo*. There is now a suite of direct elemental mapping techniques available to characterise the metallome of individual brain cells, *in situ* within *ex vivo* brain tissue sections. Such techniques include X-ray fluorescence (XRF) microscopy, [67–69] laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) [70–76], secondary ion mass spectrometry (SIMS), [77–79] and proton induced X-ray emission spectrometry (PIXE) [80–83]. The application of these elemental mapping techniques, which are direct and label free, has become an attractive analytical option for the field of neuroscience, as they can minimise or prevent distortions in metal ion homoeostasis that are known to occur during sample preparations associated with histochemical methods [84–89].

A detailed description of the techniques themselves is beyond the scope of this review, however the interested reader will find detailed descriptions of XRF, LA-ICP-MS, SIMS, and PIXE, in the literature cited herein. As a general overview: XRF offers the capability for *in situ* analysis of dehydrated (freeze-dried or air-dried) tissue sections at ambient temperature and pressure, or analysis of hydrated tissue sections (frozen) under cryogenic conditions. The method typically provides detection limits approximately at the PPM level, with spatial resolution ranging from 1–30 μm (microprobe) or 100–500 nm (nanoprobe) [67–69]. PIXE has similar capabilities to XRF, but generally the spatial resolution and detection limits are slightly poorer, and analysis under vacuum conditions is required, which prevents analysis of hydrated specimens [80–83]. LA-ICP-MS offers superior detection limits compared to XRF (PPB levels), however spatial resolution is typically on the order of 10′s of μm (1 μm is possible with state-of-the-art instrumentation, but comes at the expense of sensitivity) [70–76]. LA-ICP-MS is very well suited for scanning large sample regions with high sensitivity. SIMS offers excellent detection limits (PPM to PPB) and outstanding spatial resolution (e.g., ~ 50 nm), however the measurement is highly surface sensitive (which can be an advantage or disadvantage, depending on the application) and vacuum conditions are required [77–79].

A detailed discussion of the sample preparation considerations is not presented in this review, however there exists a range of studies that have examined in detail the effects of sample preparation on metal ion content and distribution in brain tissues [84–89]. In addition to the effects of sample preparation, careful consideration must be given when interpreting the results of indirect analyzes (e.g., detection of metal ions using histochemistry). As is the case for metal ion redistributions that can occur during chemical fixation and sample preparation, there is substantial opportunity for metal ion redistribution to occur during staining steps in histochemical protocols. Furthermore, histochemical methods (or metal-sensitive fluorescence sensors) may display heightened sensitivity to specific chemical forms of a metal ion, and insensitivity towards other chemical forms (often by design). The variability in sensitivity of detection that exists between different chemical forms of metal ions should always be thoroughly considered when interpreting the results from analyses using indirect analytical methods. The direct elemental mapping techniques discussed in this review (e.g., XRF, LA-ICP-MS, PIXE, SIMS) are advantageous as they do not show preferential sensitivity to a specific chemical form of a metal ion. However, it should also be stated that in some applications increased sensitivity towards a specific chemical form of a metal ion may offer more in-depth mechanistic insight.

Herein, this review examines current knowledge regarding the metallome of Fe, Cu, and Zn within different types of brain cells, neurons and glia, which has been determined using direct elemental mapping techniques. Specific focus is given to studies of cellular metal content determined from tissue sections, as opposed to *in vitro* cell culture.

Discussion

Zinc (Zn)

The brain is particularly enriched in Zn (~150 μM), [90] of which a substantial portion is mobile and labile (i.e., the chelatable Zn^{2+} pool) [9,90–93]. The chelatable Zn^{2+} pool has long been detected with classical histochemical stains, such as the methods developed by Timm's and Danscher [91,94–96]. In addition to histochemical detection, fluorescent Zn chelators, such as TSQ and Newport Green (to name a few) have been developed to detect labile Zn^{2+} [92,97]. The chelatable Zn^{2+} pool is released during neurotransmission from the synapses of a subset of excitatory glutamatergic neurons ("zincergic" neurons) [6,9,98]. The zincergic neurons are enriched in specific brain regions, including the CA3 sector of the hippocampus, layer II/III and V of the cortex, and olfactory bulb [90,91]. The exact chemical pathways modulated by chelatable Zn within zincergic neurons remains unknown, as does the specific chemical form of labile Zn. However, depletion of chelatable brain Zn, either by dietary induced Zn deficiency, or via disease pathologies, is associated with cognitive impairment [98]. Likewise genetic manipulation (Zn-transporter 3 knockout, ZnT3-KO) depletes chelatable Zn in the hippocampus and induces memory deficits that resemble facets of dementia [15,93]. Interestingly, while much research attention in the field of Alzheimer's disease has focussed on the accumulation of Zn in amyloid-β plaques, a recent study used elemental mapping to demonstrate that the neuropil in the CA3 mossy fibre sub-region of the hippocampus becomes depleted in Zn, adjacent to Zn enriched plaques [52]. This finding provides additional evidence through which amyloid plaques may effect cognitive function during Alzheimer's disease (i.e. through depletion of the local labile Zn pool).

Direct elemental mapping has made valuable contributions to our understanding of the chelatable Zn pool, including a seminal study in which synchrotron radiation XRF was used to quantify the magnitude of hippocampal Zn depletion that occurred in ZnT3-KO mice [93]. More recently, synchrotron radiation XRF has been used to identify depleted Zn within the tissue region that contains the synaptic terminals of the CA3 zincergic neurons (the "mossy fibres"), in an animal model of accelerated ageing [99]. Although synchrotron radiation XRF probes used in that study did not have sufficient spatial resolution to resolve individual synapses, the fact that the mossy fibre region contains almost exclusively synapses is strong evidence for synaptic Zn depletion. Possibly of little surprise, the cell body (soma) of CA3 pyramidal neurons has also been confirmed to be particularly enriched in Zn, relative to adjacent CA2 and CA1 pyramidal neurons (Fig. 1) [100]. In addition,

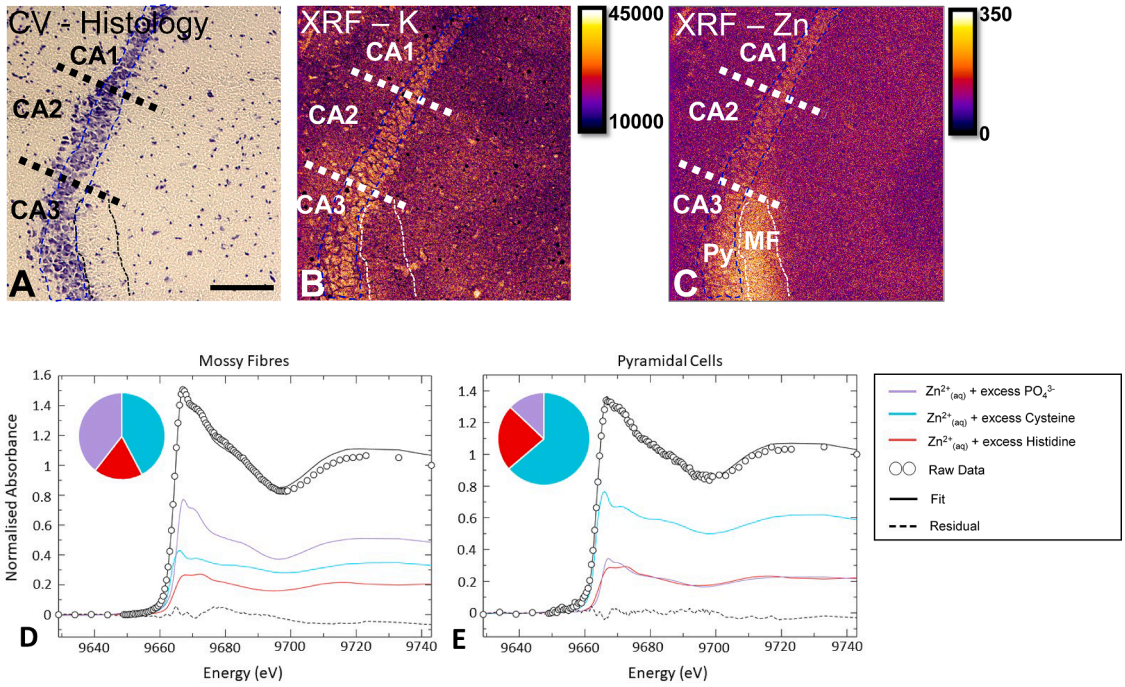

Fig. 1. Imaging the Zn metallome in brain neurons. (**A**) Cresyl violet histology showing the hippocampal pyramidal neuron cell layer (blue dashed region), which can be divided into 3 sub-regions, containing phenotypically different pyramidal cells (CA1, CA2, CA3). The dendrites of the CA3 pyramidal cells are enriched in Zn, and this anatomical location is known as the "mossy fibres" (black or white dash region). (**B**) K elemental map, collected using synchrotron radiation XRF, allows visualisation of individual cells. (**C**) Zn elemental map indicates the abundance of Zn within the mossy fibre region. Panels A-C are on the same scale, scale bar in *A* = 100 μm. Py = pyramidal cell, MF = mossy fibres. Areal density units for panel B, C are ng cm^{-2}. (**D**) X-ray absorption near edge structure spectroscopy (XANES) can be used in combination with XRF, to determine the chemical form of Zn in specific brain regions, such as the mossy fibres, or **E**) the pyramidal neurons. Panels A-C adapted with permission from reference 100. Panels D, E adapted with permission from reference 101.

recent work using X-ray absorption near-edge structure (XANES) spectroscopy has revealed distinctive differences in the chemical form of Zn found in the neuron cell body (soma) compared to tissue regions containing synapses [101]. Given the important role of chelatable Zn in memory function, and the fact that depletion in chelatable Zn is associated with cognitive decline, there has been much interest in modulating or restoring Zn pools in the brain [46]. Studies using direct elemental mapping with XRF or LA-ICP-MS have made several important contributions by revealing that various therapeutic compounds restore Zn across brain regions [46,47]. There is much potential for future studies to employ elemental mapping techniques (XRF, LA-ICP-MS, SIMS, PIXE) in combination with techniques that reveal Zn speciation (e.g., XANES) [101–103], to greatly improve our understanding of the chemical mechanisms through which Zn modulates brain function (as highlighted in Fig. 1).

Copper (Cu)

Cu in Astrocytes – Cu is an essential trace element for a multitude of biochemical pathways in the brain, where it is incorporated into enzymes or serves as a critical cofactor to support: metabolism (Cytochrome c oxidase), anti-oxidant defence (super-oxide dismutase) and neurotransmitter synthesis (Dopamin-b-monoxygenase) [11,104]. The import, storage, and subsequent export or transport of Cu ions is vital to healthy brain function, and astrocytes (from the glial cell lineage) are central to these roles [104]. The "end-feet" of astrocyte processes form a key component of the blood-brain barrier (BBB), which enables astrocytes direct control of Cu entry into the brain across the BBB [105]. Astrocytes and another specialised class of glial cells, ependymal cells, line the ventricle walls of the brain, and form a key component of the brain – ventricle barrier, which is also responsible for Cu import / export in the brain [105]. Consistent with *in vivo* roles for Cu import and storage, astrocytes have remarkable capacity to safely sequester Cu ions *in vitro* [104].

Direct elemental mapping techniques have provided valuable information on the Cu levels within astrocytes, highlighting how astrocyte Cu homoeostasis changes during ageing [106,107]. One would expect that with a prominent role in Cu import and storage, astrocytes would be enriched in Cu, and indeed this has been directly confirmed using elemental mapping [100,106–110]. Multiple research groups have used synchrotron radiation XRF to demonstrate Cu enrichment in astrocytes and ependymal cells of the brain lateral ventricles, and also Cu enrichment within the corpus callosum white matter, in mice, rats, and human tissues (as shown in Fig. 2) [100,106–111]. Studies by Pushkar et al., used an especially elegant approach of multi-modal XRF and immuno-histochemistry, to convincingly show Cu enrichment in a subset of astrocytes [106,107,109]. Further, the works by Pushie et al., [111] highlight a role for Prion Protein in brain Cu homoeostasis, with elevated Prion Protein expression resulting in increased Cu levels along the ependymal cell lining of the lateral ventricle wall. Interestingly, but perhaps not surprisingly, Cu transport and storage in the brain is not static, and Cu accumulation is observed in glial cells during ageing, concomitant with decreased Cu transport into the brain [104,105]. LA-ICP-MS in combination with ^{67}Cu auto-radiography played a key role in identifying the association between total brain Cu accumulation during natural ageing and decreased Cu import into the brain [74].

The physiological consequences of Cu accumulation within astrocytes during ageing, on a background of decreased brain-Cu import remain unknown however, impacts on SOD-1 function, brain metabolism, and neurotransmitter synthesis seem likely. Further studies are now needed, and indeed are currently underway in many groups, to elucidate the specific biochemical pathways perturbed by age-related alterations to astrocyte Cu homoeostasis.

Cu in Neuron Synapses and Dendrites – The most prevalent transition metal ion found in synapses and involved in neurotransmission is Zn^{2+}, as already discussed, however there is also mounting evidence for

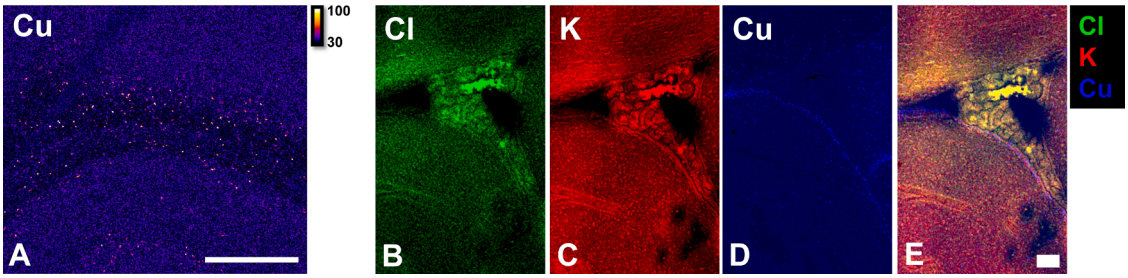

Fig. 2. Localising the Cu metallome within astrocytes. (**A**) A representative example of Cu enrichment within cells of the corpus callosum, which are most likely astrocytes, as proven by other studies.[106–109] (**B-E**) Cu enrichment is also observed in the ependymal cell layer of the lateral ventricles, which can be seen with a comparison of **B**) Cl distribution, **C**) K distribution, **D**) Cu distribution, and **E**) overlay. Scale bar in $A = 500$ μm. Scale bar in $E = 100$ μm. Areal density units for panel A is ng cm^{-2}.
Panel A is adapted with permission from reference 100. Panels B-E are adapted with permission from reference 110.

important roles of Cu ions in neurotransmission [7,112]. A number of authors have provided indirect evidence of a labile Cu^{+} pool (detected with fluorescence metal sensors), which increases in concentration following neuro-stimulation [7]. Direct elemental mapping with synchrotron radiation XRF has revealed distinct Cu enrichment within the dentate gyrus molecular layer of the hippocampus, a tissue region rich in the dendrites and synapses of dentate gyrus granule neurons (as shown in Fig. 2) [52,113]. At this stage, elemental mapping of this tissue region has only been performed at the micron level, and sub-micron resolution will be needed to resolve the Cu to individual dendrites in tissues. The elemental maps do however, help support that Cu may have important roles in synaptic function. In addition, although Cu distributions in dendrites are not yet known in brain tissue, elemental mapping of cultured neuronal cells has revealed Cu enrichment in dendritic processes [114], providing further evidence that supports involvement of Cu in regulating neurotransmission.

Iron (Fe)

Fe in Oligodendrocytes and Astrocytes – Very few studies have used elemental mapping techniques to characterise the Fe content of glial cells, such as oligodendrocytes and astrocytes. However, there is one detailed investigation that has characterised the Fe content of oligodendrocytes, astrocytes, microglia, and neurons, using PIXE [83]. The results revealed oligodendrocytes are the most Fe enriched class of brain cell, with approximately 30% more Fe than the other cell types [83]. This finding is in excellent agreement with the result of Perl's histochemical Fe staining, and Ferritin immuno-histochemistry, which have shown oligodendrocytes are especially Fe rich [48,49]. The abundance of Fe within oligodendrocytes has been linked to the high metabolic rates required for myelin synthesis [52]. Astrocytes play an important role in Fe import and transport in the brain (as they do for Cu, as already described), which most likely originates from the intimate contact between astrocyte foot processes and brain blood-capillaries. The study by Reinert et al., also suggests Fe enrichment in astrocytes [83], but not to the same extent as oligodendrocytes, which is consistent with the known fact that astrocytes do not have the same rate of oxidative metabolism as oligodendrocytes, nor do astrocytes synthesise myelin [115]. The results of Reinert are in agreement with a nano-SIMS investigation of Fe distribution in brain tissue [77]. Although the nano-SIMS study did not differentiate between astrocytes and oligodendrocytes, Fe enrichment was observed within glial cells [77]. One important consideration when interpreting the results of Reinert et al. is the fact that formalin-fixed tissues were used. It is well established that chemical fixation can result in metal ion contamination of the sample [87], in addition to redistribution or leaching of labile or mobile metal ions [85,87,89]. Therefore, the contribution of the labile metal ion pool to total metal content will likely not have been accurately captured in the studies by Reinert et al.

Fe within Pyramidal Neurons – Pyramidal neurons are a major neuron type found throughout the brain cortical layers, and within key brain structures such as the hippocampus. Interestingly, pyramidal neurons are typically thought to contain relatively low levels of Fe, as they often do not show strong Perl's staining [49,116]. However, it has now been shown that there is a substantial portion of intracellular neuronal Fe, at least within hippocampal pyramidal neurons, which can be detected with elemental mapping (as shown in Fig. 3), or modified histochemical methods using minimum exposure of tissues to fixatives, acids, and solvents [116]. These results support that neurons do contain a substantive intracellular Fe pool. This is not particularly surprising due to the fairly high metabolic requirements of neurons associated with neurotransmission, and the requirement of Fe as a co-factor for synthesis of several neuro-transmitters. There is evidence of systematic variation in neuronal Fe levels as a consequence of anatomical location within the hippocampus [100]. Specifically, CA1 pyramidal neurons located in the medial portions of the rodent (mouse and rat) hippocampus, which are more excitatory, contain greater Fe levels than neurons located in lateral regions of the hippocampus [100]. Interestingly, multiple sub-cellular PIXE elemental mapping studies have revealed the presence of elevated Fe in neuron nucleoli [82,83]. The studies analyzed chemically fixed tissues, so it remains to be demonstrated that the nucleoli are Fe-enriched *in vivo*, but further investigation into the role of Fe in neuronal nucleoli function is certainly warranted and intriguing.

Fe within Dopaminergic Neurons – The substantia nigra is a unique brain region, responsible for regulating motor movement. Neurons within the substantia nigra are typically characterised by being highly pigmented (high melanin content) and dopaminergic (synthesis and release of dopamine). A host of analytical methods have now demonstrated the importance of Fe within dopaminergic neurons of the substantia nigra – specifically, elemental mapping studies have made important contributions localising Fe to dopamine rich vesicles [22,23,114,117,118]. Dysregulated Fe homoeostasis has been implicated with pathology of dopaminergic neurons within the substantia nigra, particularly in Parkinson's disease [22,23,114,117,118].

Fe within Inter-Neurons There has been little research attention given to date on the metallome within interneurons, such as the granule cells of the hippocampal dentate gyrus, or the cerebellar granule cells. Interestingly, in published elemental maps from rats but not mice hippocampal tissues, one can often observe the location of the dentate gyrus as a region of elevated Fe content [46,52,100,108]. This suggests possible species differences in the metallome of interneurons, which requires further study.

Fe in Microglia – Perl's histochemistry frequently shows Fe deposits within microglia, the brains resident immune cells [48,49]. Subsequent immuno-histochemistry confirms that microglia can store large reserves of Ferritin, [119–121] which likely accounts for the Perl's positive staining. Not unexpectedly, substantial Fe content is observed within microglia in elemental mapping of fixed tissue sections [83]. Following

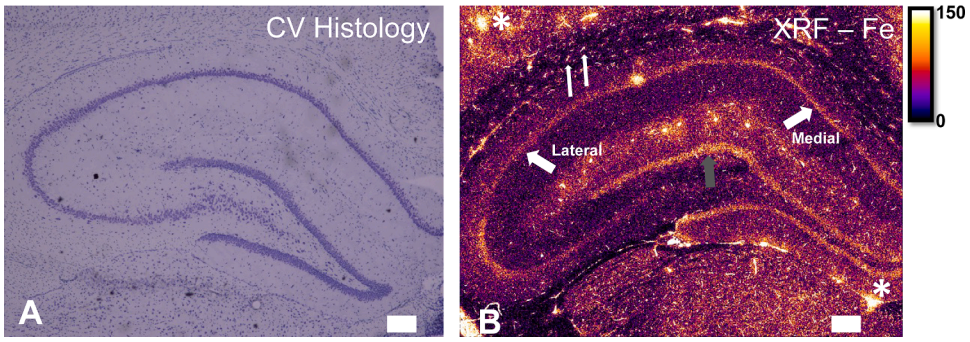

Fig. 3. An example of imaging the Fe metallome in the rat hippocampus. (A) Cresyl violet histology reveals the anatomical structure of the hippocampus. (B) An XRF elemental map of Fe distribution in the hippocampus and surrounding structures. XRF reveals an abundant pool of Fe in hippocampal pyramidal neurons, with medial neurons containing more Fe than lateral neurons (thick white arrows). The dentate gyrus granule cells (grey arrow) also appear Fe enriched relative to surrounding tissue. Small, highly Fe enriched cells can be seen in the corpus callosum white matter, which are most likely oligodendrocytes (thin white arrows). White asterisks in top left and bottom right of the image indicate blood vessels. Scale bar = 100 μm. Areal density units for panel B are ng cm^{-2}. Panels A, B adapted with permission from reference 100.

tissue injury or neurodegeneration there is often a strong inflammatory response and recruitment of microglia and other Fe rich inflammatory cells (e.g., macrophages) to the site of tissue damage. On the basis that inflammatory cells are enriched in Fe, one would therefore expect elevated Fe content at or surrounding the site of tissue damage, and indeed this has been demonstrated in conditions such as ischaemic stroke, [37] haemorrhagic stroke, [39] multiple sclerosis, [26] and traumatic brain injury [34].

Conclusions and future perspectives

There is now substantial evidence that metal ions are essential for healthy brain function, and disturbed brain metal homoeostasis is implicated in neurodegeneration and altered cognitive function. Unfortunately, our understanding of the brain metallome lags behind that of the genome, proteome, or metabolome. Nonetheless, with increased technique development and greater access to elemental mapping techniques such as XRF, LA-ICP-MS, PIXE, and nano-SIMs, the field is now well positioned for in-depth study and characterisation of the brain metallome. In particular, greater understanding of the sub-cellular localisation of metal ions within specific cells *in situ* within tissue sections, is urgently needed. It is anticipated that the increasing availability of nano-probes will provide the tools to address this knowledge gap in the near future. This review has aimed to summarise the current knowledge of differences in the metallome (Fe, Cu, Zn) of different types of brain cells (astrocytes, oligodendrocytes, microglia, and neurons), and it should be clear from this literature that there are distinct differences in the intracellular metallome between different brain cells. There is now need for detailed fundamental studies to examine exactly how natural brain physiology influences the brain metallome, in addition to elucidating how the metallome within specific brain cells helps maintain brain health. Provision of such information may then help identify precisely how metal ions are involved in the development and progression of neurodegenerative diseases.

Declarations of Competing Interest

The authors declare no conflicts of interest.

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