Analysing the Impact of Iron Dysmetabolism on Regional Metal Ion Distribution in the Brain

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INTRODUCTION

'Trace metals in medicine' have become of increasing interest in recent years especially with the study of neurodegenerative diseases like Alzheimer's (AD) and Parkinson's (PD). Certain metals protect the brain against disease but the balance of these metals is precise[1]. Abnormal metal deposits have been found in patients with AD and PD, particularly with iron elevation[2]. To investigate the relationship between iron and neurodegenerative diseases, mouse models have been developed to explore causes of iron accumulation in the brain. The H-Ferritin Deficient Model developed by Thompson et al (2003) causes a deficiency in the iron storage protein Ferritin. Furthermore, as haemochromatosis has been linked to AD[3], an Iron Overload condition was created through mouse diet manipulation. A method for comparing metal ion concentrations between these models and a control group was needed, as disease-related changes in various metal ion concentration in specific brain regions have been linked to neurodegenerative diseases[4]. Traditional methods of mapping free metals in the brain involves staining, however this doesn't allow for simultaneous multi-element analysis or metal quantification[5]. This study used Synchrotron Microfocus X-ray Fluorescence Spectroscopy (μXRF) which allowed semi-quantitative mapping of brain metals at a high resolution. The whole brain was mapped with regions being selected based on identifiable metal ion differences post-imaging. This 'backwards' approach allowed for a thorough and objective data analysis. Results were then placed in context: for example, in PD, iron decreases in Globus Pallidus, copper decreases and zinc increases in the Substantia Nigra[6], and iron appears largely unaffected in the H-Ferritin Deficient condition[1].

SUMMARY

- An Iron Overload and an H-Ferritin Deficient Mouse Model were used to examine the impact of disrupted iron metabolism on the brain.
- Brain sections were imaged and compared using Synchrotron μXRF spectroscopy. Quantitative measurement of the relative metal ion concentrations for iron, copper and zinc were made across selected regions of interest in the brain.
- It was generally found that metal ion concentrations of iron and zinc decreased in specific regions in the Iron Overload condition compared with the control, with copper increasing in only one region. Few regions differed in metal ion concentration between the H-Ferritin Deficient Model and the control.
- The three conditions exhibited similar / identical results for metal ion concentrations in many brain regions, indicating the validity of the method used for comparison between samples. It is clear that there exists a complex relationship between these trace metals. Further work is needed to test the significance of the findings in a larger sample size.

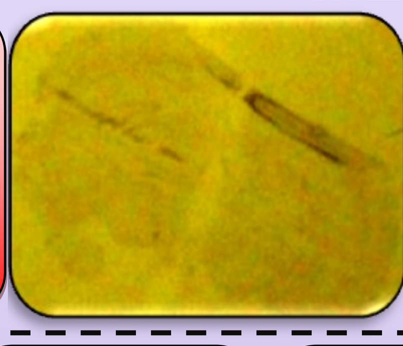
METHODOLOGY

Pre-Project

Mice Conditions 1) H-Ferritin Deficient 2) Iron Overload

Brains were removed and frozen at 7 months by Prof.J.Connor's Group at Penn State University. Frozen brains were sectioned coronally at 30μm thickness onto ultraclean quartz slides and cover-slipped with kapton

Images of the slides were taken under the light-microscope for later reference



The sections were taken to the Diamond Light Source Synchrotron, beamline I18, where they were imaged using Synchrotron μXRF

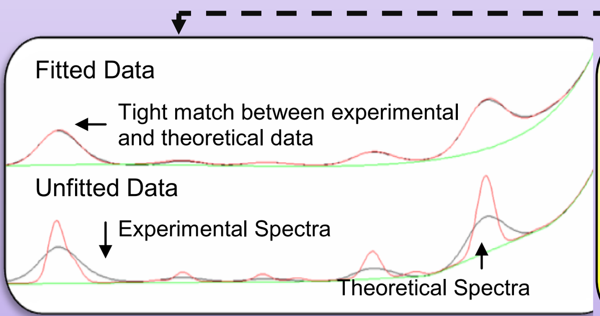
Each section was imaged at 10keV with a 60μm spot size. Exposure rate was 1 second per point in each

The μXRF Spectrum at each point was collected: this contained intensity information for each of the elements present

URSS Project Data Analysis

Post-Synchrotron measurement: the data were converted into PyMca software[8] compatible format

The experimentally measured fluorescent spectral peaks were batch-fitted with the theoretical position of the individual elements

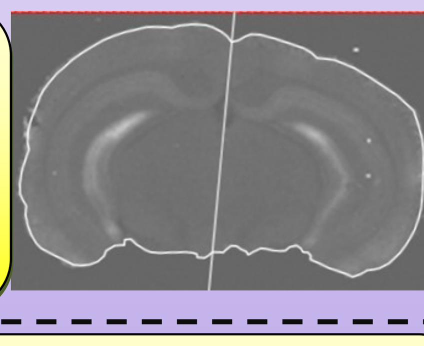


Data were normalised to the incoming beam intensity used during fluorescence mapping and then exported as .tiff files

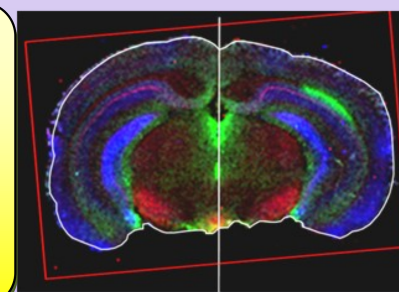
.Tiff images for each element of interest contained pixels with absolute intensity values

The .tiff files were imported into PowerPoint where they were scaled to 400% whilst maintaining their aspect ratio

A boundary template was drawn around the rectangular scanned area and another around the tissue section

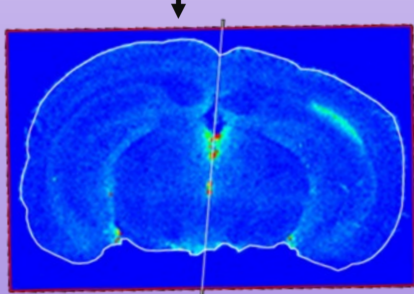


A centre line was drawn using the copper maps as reference

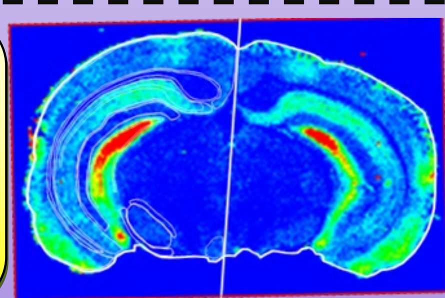


The metal ion distribution maps were scaled and rotated to fit the boundary template exactly

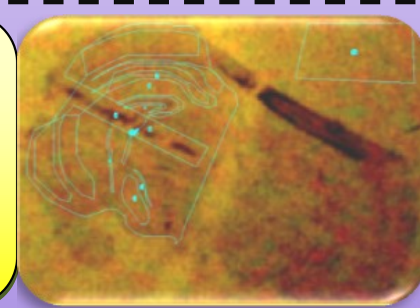
Each .tiff image was imported into PyMca and saved as colour-intensity (temperature) maps which represented the metal concentration distribution across the section



Boundaries were drawn around each identifiable brain region on the different maps. By varying the intensity scales, different regions became visible for template creation and were added to the template. This cumulative process led to the development of the final template with each relevant region outlined

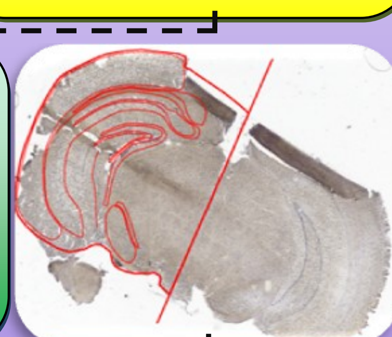


To quality-check the templates, they were overlaid onto the light-microscope images to check for cracks or folds in the original sections. Adjustments were made to ensure no faulty areas were included



Validation

Sections were stained using haematoxylin to highlight brain region boundaries (seen by changes in cell density) for validation of the templates made from the metal ion maps

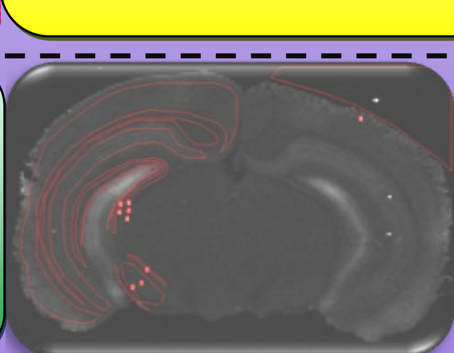


The regions outlined in the template were colour filled for clarity of region boundary after scaling

Templates were imported into ImageJ, (open source image processing software) where they were scaled to correspond to the original data sets for direct overlay

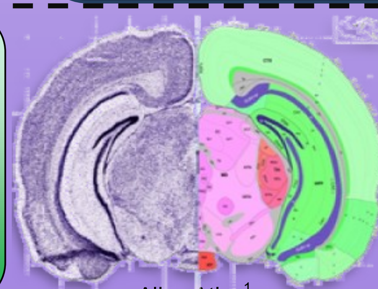
Template boundaries were traced using ImageJ's ROI selection tool. These regions of interest were then saved

The saved ROIs were used to measure intensity of each element by brain region



Mean, Standard Deviation and Histogram data for each region were measured using ImageJ and recorded in Excel

Each region was cross-referenced with the relevant level in the Allen Mouse Brain Atlas



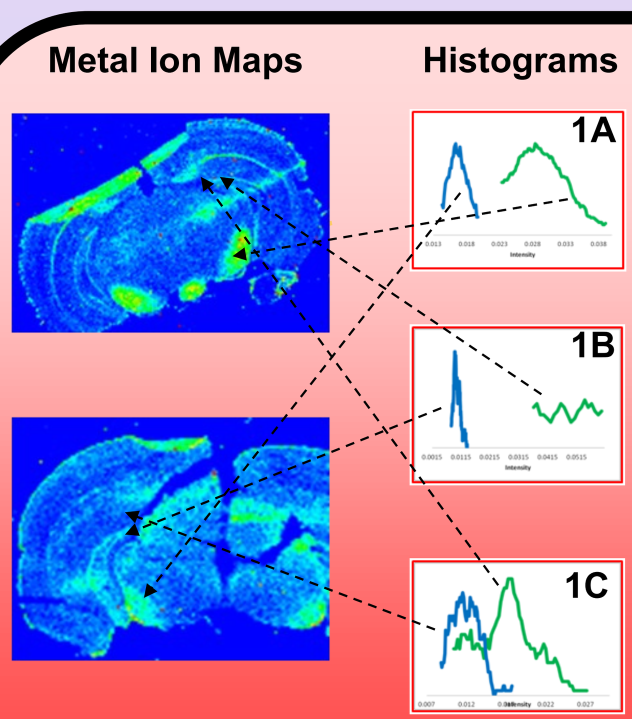
Allen Atlas[7]

Radar plots were drawn with the standard deviation acting as 'error' bars for each absolute mean measurement taken for each region and condition. Notable differences are recorded in **RESULTS**

Key: Red stages— sample preparation and imaging. Completed before the URSS Project commenced. Yellow and Green stages were completed during the URSS Project: Yellow: data processing and templating, Green: template validation and metal ion analysis.

RESULTS

IRON



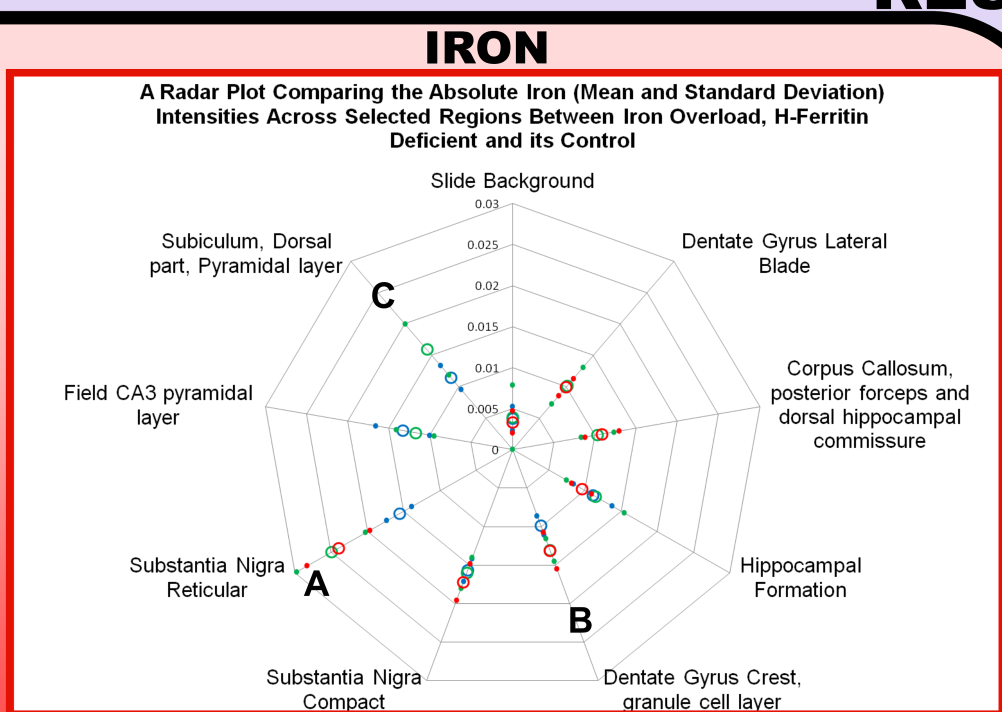


Fig 1

COPPER

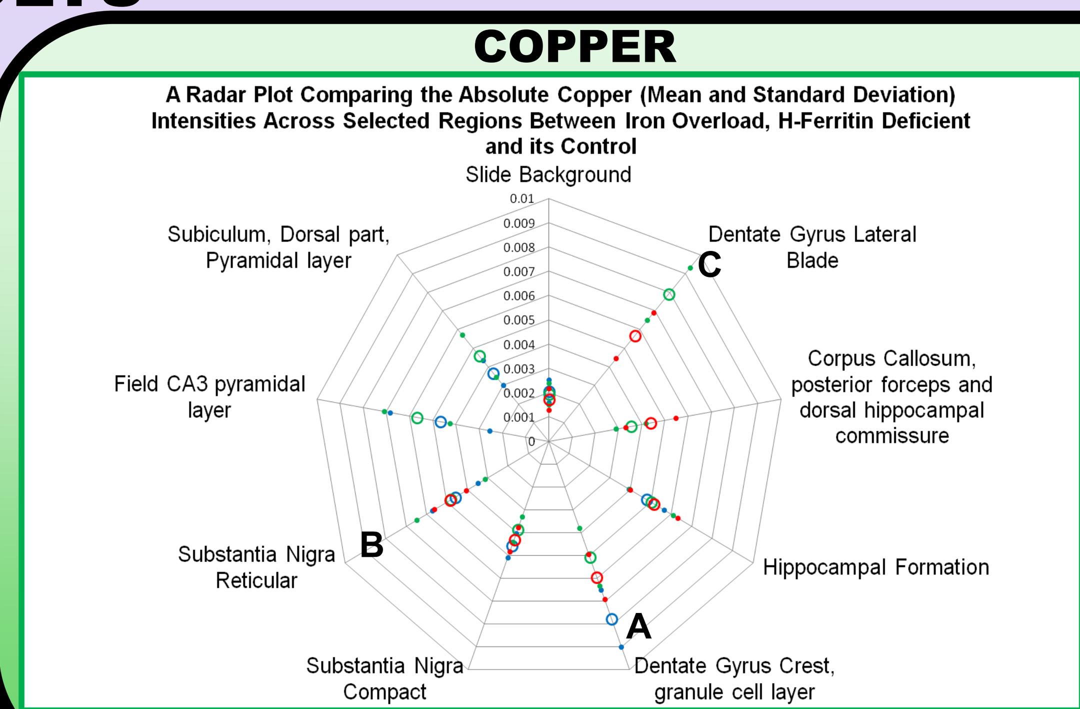
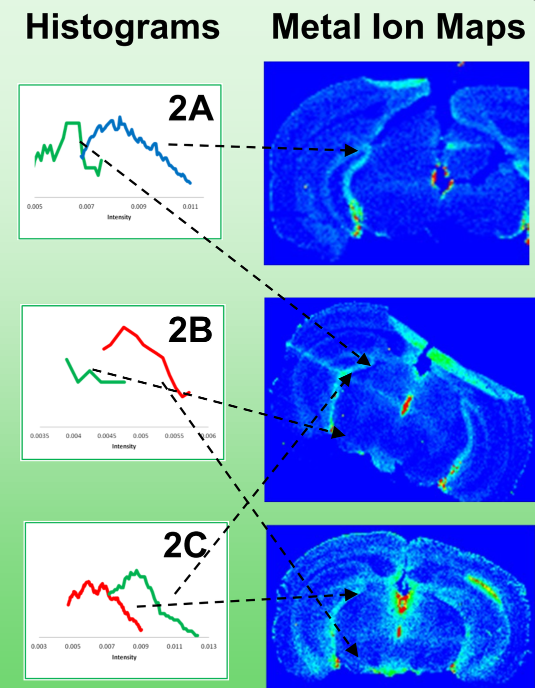
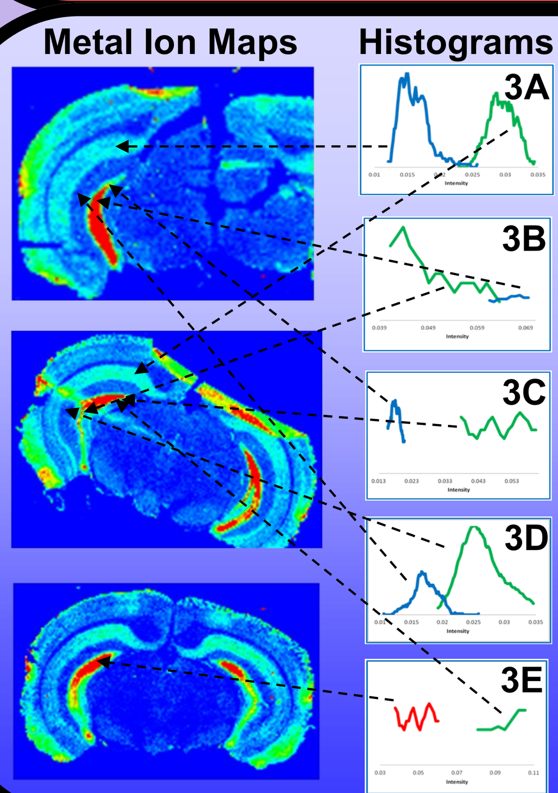


Fig 2



ZINC



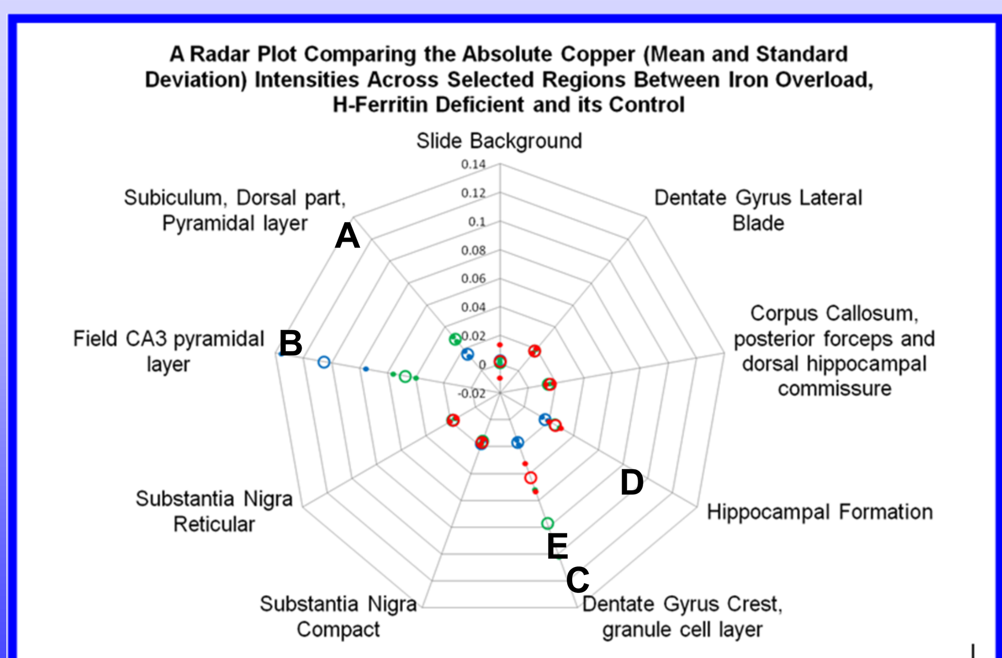
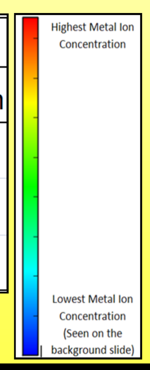


Fig 3

RESULTS OVERVIEW

Noteable differences in Metal Ion Concentration compared to Control Condition				
Mouse Condition	Brain Region	Metal Ion: Iron	Copper	Zinc
Iron Overload	Hippocampal Formation			↓
	Dentate Gyrus Crest	↓	↑	↓
	Field CA3 pyramidal layer			↑
	Substantia Nigra Reticular	↓		
	Subiculum, Dorsal part	↓		↓
H-Ferritin Deficient	Dentate Gyrus Lateral Blade		↓	
	Field CA3 pyramidal layer			↓
	Substantia Nigra Reticular			↑

Histogram Key	
O,●	Mean, Standard Deviation
O,● (blue)	Iron Overload
O,● (red)	H-Ferritin Deficient
O,● (green)	Control - Wild Type



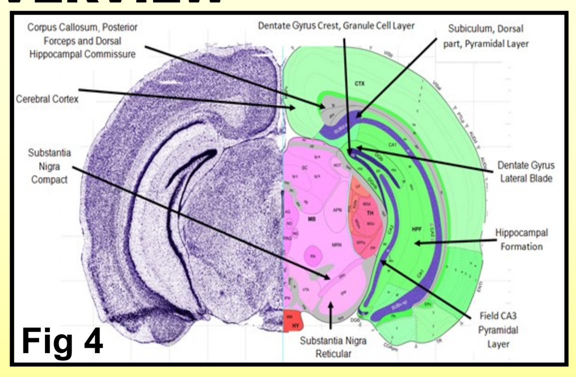


Fig 4

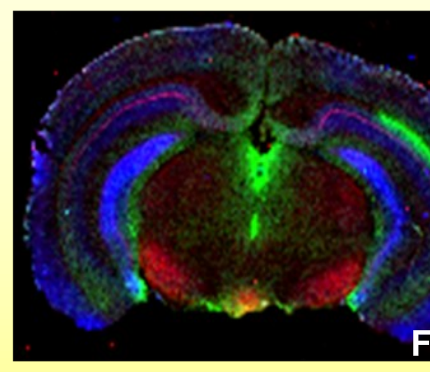


Fig 5

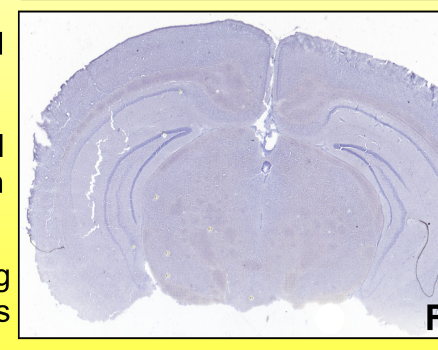


Fig 6

Fig 4: Allen Mouse Brain Atlas level corresponding to mapped regions[7]

Fig 5: Metal ion map showing the metal distribution throughout all regions for Iron (red), Copper (green) and Zinc (blue)

Fig 6: A stained tissue section showing the different cell densities and types across brain regions

CONCLUSIONS and FURTHER WORK

1) We observed strong regional variations in brain metal ion concentrations (Fig 5); some of these are consistent with evidence in the current research literature.
2) The concentration range within a given region varies as a function of metal element and brain region as shown by the histograms (Figs 1A-C, 2A-C and 3A-E).
3) Disrupting the iron metabolism affects zinc and copper as well as iron across different regions, indicating a relationship between these metal ions.
4) For the Iron Overload model, iron decreases in the Dentate Gyrus Crest, Subiculum and also the Substantia Nigra Reticular, compared to the control.
5) It was found that iron concentration doesn't change in the H-Ferritin Deficient condition which agrees with the literature[1], but zinc does increase in the Substantia Nigra Reticular which is similar to PD[6].
6) The narrow distribution of intensities and similarity of concentration measured in the majority of brain regions and from the slide background (Figs 1-3) indicate that the method for sample comparison is valid.
7) A larger sample size is required to test for statistically significant differences between the three groups.

ACKNOWLEDGEMENTS

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REFERENCES

1 **Thompson**, K., et al (2003) Mouse Brains Deficient in H-Ferritin Have Normal Iron Concentration but a Protein Profile of Iron Deficiency and Increased Evidence of Oxidative Stress. *Journal of Neuroscience Research*, 71, pp.46-63

2 **Becker**, J.S., et al (2009) Bioimaging of Metals by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). Mass Spectrometry Reviews, 29, pp.156-175

3 **Connor**, J.R., et al (2001) Is Hemochromatosis a Risk Factor for Alzheimer's Disease? *Journal of Alzheimer's Disease*, 3, pp.471-477

4 **Pinero**, D.J., Connor, J.R. (2000) Iron in the Brain: An Important Contributor in Normal and Diseased States. Neuroscientist, 6, pp.435-453

5 **Punshon**, T., et al (2005) Application of Synchrotron X-Ray Microbeam Spectroscopy to the Determination of Metal Distribution and Speciation in Biological Tissues. Spectrosc Lett, 38, pp.343-363

6 **Dexter**, D.T., et al (1991) Alterations in the Levels of Iron, Ferritin and Other Trace Metals in Parkinson's Disease and Other Neurodegenerative Diseases Affecting the Basal Ganglia. Brain, 114, pp.1953-1975

7 **Allen Mouse Brain Atlas** [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Available from: http://mouse.brain-map.org.

8 **Solé**, V.A., et al (2007) A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. *Spectrochim*, 62, pp.63-68.