THE RELATIONSHIP OF IRON AND AMYLOID: INSIGHTS FROM A NEW MOUSE MODEL OF IRON LOADING AND AMYLOIDOSIS

Ritambhara Aryal

B.Sc. Biotechnology

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

MARCH 2019

FACULTY OF HEALTH

UNIVERSITY OF NEWCASTLE

This research was supported by an Australian Government Research Training Program (RTP) Scholarship

Statement of originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Ritambhara Aryal

Acknowledgements

It is hard to believe that the four-year long roller coaster ride with my thesis has finally come to a beautiful end. Words are not enough to thank everyone who has supported my PhD journey, all this time. Firstly, I would like to express a humble gratitude to my principal supervisor, Prof. Liz Milward. The collaboration that started off with some snorkelling lessons has now resulted into the successful completion of this research project. Needless to say, Liz's supervision has been remarkable not only for my PhD project but also for my professional and personal development.

I am thankful for the financial assistantship that I received through the Australian Government Research Training Program Scholarship. I would also like to give thanks to my co-supervisor Dr Dan Johnstone, who was always willing to provide valuable suggestions regarding my project and also helped me with my lab work at University of Sydney. I am thankful to the staff of the research support unit and teaching support unit (School of Biomedical Sciences), Dr Karen Mate and Dr Sivaraman Purushothuman for their support during my PhD.

I will always cherish the brilliant camaraderie that I developed with my labmates Kristy, Jason, Seak, Will, Sam, Elvis, Moones and Ali. They have made me realise the power of teamwork in the accomplishment of our individual research goals and in the collective upliftment of our careers.

Lastly, I dedicate this thesis to my family, which means the world to me. My earnest gratitude to Ashim, who has been my pillar of strength and my motivator at times when I am filled with self-doubt and apathy. My parents (Aama and Bauji) have constantly taught me to do best in all of my endeavours and I am really grateful to them for believing in my ability, more than I do myself. I am sure that Supu, my little one will be super proud of this accomplishment, just as she has always been even in my tiniest of achievements. Lastly, I am thankful to my extended family members (Mamu, Buwa, Agya didi and Suraj bhinaju) who have always inspired me with their kind and encouraging words not to give up on my goal that I had set four years ago.

"It always seems impossible until it's done."

- Nelson Mandela

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAS	Atomic absorption spectroscopy
Aβ	Amyloid-β
AD	Alzheimer's disease
AICD	APP intracellular domain
ANOVA	Analysis of variance
APLP1	Amyloid-precursor-like protein-1
APLP2	Amyloid-precursor-like protein-2
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BACE1	β-site APP-cleaving enzyme 1
BBB	Blood brain barrier
CI	Confidence interval
CNS	Central nervous system
CP	Ceruloplasmin
CR	Congo red
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Cerebrospinal fluid
DAB	3, 3'-diaminobenzidine
Dcytb	Duodenal cytochrome b
ddH_2O	Deionised, distilled water
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DPX	Di-n-butylphthalate in xylene
ELISA	Enzyme-linked immunosorbent assay

EOAD	Early onset Alzheimer's disease
ES	Embryonic stem
FAD	Familial Alzheimer's disease
Fe2+	Ferrous iron
Fe3+	Ferric iron
Fpn	Ferroportin
FTIR	Fluorescence
GFAP	Glial fibrillary acidic protein
GPI	Glycophosphatidylinositol
h	Hours
HAMP	Hepcidin gene
HCP1	Haem carrier protein 1
HEK293T	Human embryonic kidney 293
Heph	Hephaestin
Hfe KO	*Hfe* knockout mice
H-ferritin	Ferritin heavy chain
HH	Hereditary haemochromatosis
HJV	Hemojuvelin
HO	Haem oxygenase
H_2O_2	Hydrogen peroxide
HRP	IgG-horseradish peroxidise
IHC	Immunohistochemistry
IRE	Iron responsive element
IRP	Iron regulatory protein
KO	Knockout

L-ferritin	Ferritin light chain
LIP	Labile iron pool
LOAD	Late onset Alzheimer's disease
MAPT	Microtubule-associated protein tau
min	Minutes
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRI	Magnetic resonance imaging
N	Experimental sample size
NFTs	Neurofibrillary tangles
nm	Nanometer
NS	Not significant
NTBI	Non-transferrin-bound iron
OCT	Optimal cutting temperature
OD	Optical density
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween
PHFs	Paired helical filaments
PS/PSEN	Presenilin
PS1	Presenilin 1
PS2	Presenilin 2
p-tau	Phospho-tau
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
rpm	Revolutions per minute

RT	Room temperature
s	Seconds
SEM	Standard error of the mean
sAPPα	Soluble APPα
STEAP	Six transmembrane epithelial antigen of the prostrate
TBI	Transferrin-bound iron
Tf	Transferrin
TFR	Transferrin receptor
TFR1	Transferrin receptor 1
TFR2	Transferrin receptor 2
Tfr2mut	*Tfr2* mutant
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TX-100	Triton X-100
UTR	Untranslated region
WT	Wildtype control mice

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease which has been proposed to be associated with brain iron abnormalities, although this remains contentious. To investigate the broad hypothesis that increased brain iron levels may exacerbate Alzheimer's amyloid pathology, this project studied a novel mouse model (the 'Aβ+Iron model'). This model was developed by cross-breeding the APPswe/PS1ΔE9 mouse model of amyloidosis (the 'Aβ model') with the *Hfe*$^{-/-}$*xTfr2*^{mut} mouse model of the iron loading disorder haemochromatosis (the 'Iron model') and backcrossing onto the AKR background strain to maximize iron loading.

Brain iron content by non-haem iron assay of homogenised brain hemispheres at 6 months of age showed substantial iron loading in Aβ+Iron mice compared to age-, gender- and strain-matched Aβ mice (fold change ≥ 1.8, p<0.0001, n ≥11 mice/group, with ≥4 per sex in each group). Likewise the relative intensity of 3,3'-diaminobenzidine-4HCl (DAB)-enhanced Perls' staining for iron was significantly increased in Aβ+Iron mice compared to Aβ mice (fold change 1.7, p<0.0001, n=4 mice/group).

Since the Iron model does not express human Aβ, it would not be generally predicted to have classical amyloid. This model was used to assess whether abnormal mouse Aβ deposition could be induced by the presence of increased brain iron levels even though this model does not contain any human Aβ sequence. As expected, classical amyloid with Congo red birefringence under polarised light was not observed in the Iron model. These mice produce only endogenous murine Aβ which is not likely to aggregate and form plaques (i.e model does not express human Aβ), suggesting increased brain iron levels alone are not sufficient to induce amyloid formation in the absence of amyloid-related mutations. Histological labelling with Congo red stain for amyloid alone or in combination with DAB-enhanced Perls' stain for iron was used to examine whether increased brain iron levels altered amyloid deposits in the Aβ and Aβ+Iron transgenic models. No differences were observed for mean counts and size distribution of amyloid deposits, amyloid burden or amyloid density across matched Bregma (-2.46 to -3.16) in Aβ+Iron compared to Aβ mice (all p>0.05, n=4 mice/group). This Bregma range includes hippocampal and entorhinal cortex regions strongly affected in AD.

An increased proportion of amyloid plaques had observable iron labelling in the Aβ+Iron model, in which 99.25% of detected plaques co-localised with DAB-enhanced Perls' stainable iron compared to 81.09% in the Aβ model (p=0.0016, n=4 mice/group). Likewise, iron labelling around plaques was stronger in the Aβ+Iron model than the Aβ model, based on the ImageJ Transformed Mean Gray Value, a measure of staining intensity, of the iron halos surrounding the Congo red plaque

cores (fold change 1.4, p=0.0456, n=4 mice/group). However there was no difference in the mean area of the halos surrounding plaques between the two models (p=0.1007, n=4 mice/group).

Co-labelling was also performed for iron (DAB-enhanced Perls' stain) and Aβ peptide using antibody 4G8. This antibody, in addition to classical Aβ amyloid that is birefringent under polarised light, also detects other forms of insoluble Aβ peptide deposits. Antibody 4G8 and iron generally co-localised closely, with detectable iron usually though not always restricted to the 4G8 immunolabelled region.

In general, there were more amyloid plaques in brain areas with less iron staining compared to areas with high iron staining (correlation coefficient -0.97). For example, few if any amyloid plaques were detected in basal ganglia and thalamus with strong iron staining compared to regions such as the hippocampus and entorhinal cortex.

Overall, regional and cellular distributions of iron in the Aβ+Iron model were similar to those in the Iron model with the important exceptions that iron co-localised with amyloid plaques in the Aβ+Iron model and iron-laden cells were present in the immediate vicinity of plaques. Iron staining was most conspicuous in the choroid plexus by all methods used (DAB-enhanced Perls' stain and traditional or perfusion Turnbull stain for ferrous iron). Neurons contained very little stainable iron in any region examined, including hippocampus, cerebral cortex and midbrain. Co-labelling with DAB-enhanced Perls' stain and Luxol fast blue stain or a myelin-specific marker 2', 3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) revealed substantial amounts of iron in myelinated regions. Ferritin heavy and light chain immunolabelling co-localised with DAB-enhanced Perls' stain in a subset of myelin-associated cells with the morphology of oligodendroglial 'trains', consistent with previous literature demonstrating iron in a subset of oligodendroglia in rodents and humans.

Brain regions with numerous glial fibrillary acidic protein (GFAP) labelled astrocytes typically had few iron-laden cells, with few if any cells co-labelled for both GFAP and iron. Likewise regions with increased iron accumulation typically contained few astrocytes, suggesting there was little reactive astrogliosis in areas with increased iron accumulation, and little if any reactive astrogliosis was observed around amyloid plaques.

The proportion of plaques surrounded by iron-laden cells resembling activated amoeboid microglia or microglia transitioning into activation states was much greater in the Aβ+Iron model compared to the Aβ model (22% as opposed to 77%), suggesting iron may exacerbate microgliosis in response

to Aβ. This is potentially important since microglial activation appears to facilitate clearance of amyloid, although excessive accumulation of iron could eventually wind up damaging or killing microglia and weakening the brain's defensive responses.

Additional studies are required to investigate these possibilities since few if any cells co-labelled for both iron and the ionized calcium-binding adapter molecule 1 (Iba1), a marker for microglia and while Iba1 positive microglia were present in the immediate vicinity of iron-amyloid complexes, these did not co-label for iron, although reduced sensitivity of double and triple labelling procedures cannot be ruled out. There was no difference in the average count of Iba1-positive microglia in the vicinity of plaques between the Aβ+Iron and Aβ models (p=0.4073, n=4 mice/group).

There was limited preliminary evidence of oxidative damage in the Aβ+Iron model. While no labelling was detected in any model for 8-hydroxy-2'-deoxyguanosine (8-OHdG), which detects DNA oxidation damage, there was some putative positive but very weak immunolabelling in all models for lipid peroxidation damage as assessed by 4-hydroxynonenal (4-HNE) antibody that appeared slightly stronger in the Aβ+Iron and Aβ models but this also needs to be confirmed in further studies.

Increased levels of iron did not seem to increase neuronal loss in preliminary studies with neuronal nuclear (NeuN) antibody labelling. Specifically there was no significant decrease in relative neuronal counts per unit area at matched Bregma in the Aβ+Iron model compared to the Aβ model in the full cerebral hemisphere, excluding the cerebellum (p=0.3331, n=4 mice/group, one-tailed t test).

In summary, these results confirm that brain iron levels are increased in the Aβ+Iron model at 6 months of age and that iron co-localises with amyloid in this model but does not appear to affect measures of amyloid load. Although there was some preliminary evidence of lipid peroxidation damage and increased levels of ferrous iron in a few areas with high levels of iron by DAB-enhanced Perls' staining, amyloid formation was usually not observed in these regions and no neuronal death was observed across the cerebral hemisphere.

Several protective mechanisms may be involved. Most iron appears to remain sequestered within myelin, oligodendroglia or other unidentified glia, with neurons containing little if any Perls' stainable iron. Cells morphologically resembling transitional or activated amoeboid microglia appear to take up iron in the vicinity of amyloid plaques and may also have protective roles but these were not confirmed to be microglia by Iba1 labelling and remain unidentified.

This study has provided new insights into the nature of the relationship of iron and AD. The findings suggest that surplus iron may be safely sequestered by normal brain iron homeostatic and storage mechanisms and may not appreciably influence Alzheimer's disease pathogenesis at least in the earlier stages of the disease course corresponding to the period examined in the present study. In the light of these observations, the low levels of neuronal iron and the possibility that at least initially, iron may be important in increasing activation of microglia around plaques, facilitating amyloid clearance, iron chelation may be potentially deleterious, at least in the early stages of disease and extreme caution should be exercised before pursuing clinical trials or recommending iron chelation as a treatment for AD and other neurodegenerative conditions.