CELLULAR AND MOLECULAR CHANGES IN THE BRAIN OF THE *Hfe*$^{-/-}$*xTfr2*^{mut} MOUSE, A MODEL OF HUMAN IRON LOADING

Moones Heidari

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Statement of Originality

The thesis contains no material which has been accepted 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 in the text. 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. **Unless an Embargo has been approved for a determined period. Moones Heidari

Statement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices as follows.

My co-supervisor Daniel Johnstone from the University of Sydney has bred, housed and provided tissue from some of mice used in this study. This project has arisen out of his PhD and postdoctoral research and he has also contributed to some of the experimental data, including ICP-AES and human and mouse microarrays.

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List of Abbreviations

aco	Anterior commissure
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
ATP13a2	ATPase type 13A2
β2m	Beta-2 microglobulin
BBB	Blood-brain barrier
BMP	Bone morphogenetic protein
BPAN	Beta-propeller protein-associated neurodegeneration
CBN	Cerebellar nuclei
C19orf12	Chromosome 19 open reading frame 12
cc	Corpus callosum
cDNA	Complementary DNA
chpl	Choroid plexus
CNS	Central nervous system
CoA	Coenzyme A
Coasy	CoA synthase
CoPAN	COASY protein-associated neurodegeneration
Cp	Ceruloplasmin
CP	Caudoputamen
cRNA	Complementary RNA
CSF	Cerebrospinal fluid
Ct	Threshold cycle
DAB	3,3'-diaminobenzidine-4HCl
DAVID	Database for Annotation, Visualization and Integrated Discovery
Dcaf17	DDB1 and CUL4 associated factor 17
Dcytb	Duodenal cytochrome b
df	Dorsal fornix

DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
EGR	Early growth response
Fa2h	Fatty acid 2-hydroxylase
FAHN	Fatty acid hydroxylase-associated neurodegeneration
FC	Fold change
Fe2+	Ferrous iron
Fe3+	Ferric iron
fi	Fimbria
FLVCR	Feline leukemia virus subgroup C cellular receptor
Fpn	Ferroportin
Ftl	Ferritin, light polypeptide
Fth	Ferritin, heavy polypeptide
fxs	Fornix system
GABA	Gamma aminobutyric acid
GATHER	Gene Annotation Tool to Help Explain Relationships
gl	Glomerular layer
GP	Globus pallidus nuclei
GPI	Glycosylphosphatidylinositol
gr	Granular layer
h	Hours
Hamp	Hepcidin antimicrobial peptide
HCP1	Heme carrier protein 1
Heph	Hephaestin
HH	Hereditary hemochromatosis
HJV	Hemojuvelin
hip	Hippocampus
HO	Heme oxygenase
ICP-AES	Inductively coupled plasma-atomic emission spectroscopy
ICTX	Isocortex
int	Internal capsule
ipl	Inner plexiform layer

IRE	Iron-responsive element
IRP	Iron regulatory protein
JH	Juvenile Hemochromatosis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LFB	Luxol Fast Blue
MAPK	Mitogen-activated protein kinase
mi	Mitral layer
min	Minutes
mo	Molecular layer
MPAN	Mitochondrial-membrane protein-associated neurodegeneration
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Medial septal nucleus
mtt	Mammillary related tracts
NBIA	Neurodegeneration with brain iron accumulation
NO	Nitric oxide
NS	Not significant
NTBI	Non-transferrin-bound iron
onl	Olfactory nerve layer
opl	Outer plexiform layer
PANK2	Pathothenate kinase 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKAN	Pantothenate Kinase-Associated Neurodegeneration
Pla2g6	Phospholipase 2 group VI
PLAN	PLA2G6-associated neurodegeneration
po	Polymorph layer
pu	Purkinje layer
RN	Red nucleus
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction

RT	Room temperature
s	Seconds
SEM	Standard error of the mean
sg	Granule cell layer
slm	Stratum lacunosum-moleculare layer
SMAD	Mothers against decapentaplegic homologue
SN	Substantia nigra
so	Stratum oriens
sp	Pyramidal layer
sr	Stratum radiatum
STAT	Signal transducer and activator of transcription
STEAP	Six transmembrane epithelial antigen of the prostate
TBI	Transferrin-bound iron
Tf	Transferrin
Tfr1	Transferrin receptor 1
Tfr2	Transferrin receptor 2
TH	Thalamus
UTR	Untranslated region
Wdr45	WD repeat domain 45
WM	White matter

Abstract

Brain iron dyshomeostasis has been proposed to be associated with various severe neurodegenerative diseases such as Alzheimer's disease, although the nature of the association remains controversial. It is unclear whether brain iron accumulation and consequent degeneration are also a feature of peripheral iron loading disorders such as hereditary hemochromatosis. To help understand how systemic iron loading affects the brain, this project studied mice with disruption of two iron regulatory genes, the hemochromatosis (*Hfe)* gene and the transferrin receptor 2 (*Tfr2*) gene.

Brain iron content measured by non-heme iron assay at 3 or 9 months of age showed significant iron accumulation in the $Hfe^{-/-}xTfr2^{mut}$ mice compared with age-, gender- and strain-matched wildtype mice (fold change ≥1.9, $p<0.0005$ n≥4 mice/group). Regional iron distribution was investigated by enhanced 3,3'-diaminobenzidine-4HCl (DAB) Perls' staining, which confirmed greater iron accumulation in the brain of $Hfe^{-/-}xTfr2^{mut}$ mice at 3, 6, 9 and 12 months of age than in the brain of wildtype mice, although the regional and cellular distributions were very similar in both groups of mice. The choroid plexus was the most intensely iron-stained structure. Co-staining with DAB-enhanced Perls' stain for iron and Luxol Fast Blue stain for myelin showed that in both $Hfe^{-/-}xTfr2^{mut}$ and wildtype mice, substantial proportions of iron were co-localized in myelinated fibers and patches in the corpus callosum, internal capsule, fornix system, basal ganglia and cerebellar white matter. A few cerebellar Purkinje cells occasionally showed very low levels of iron staining that did not appear to differ between $Hfe^{-/-}xTfr2^{mut}$ and wildtype mice. No other neurons, including hippocampal neurons, were observed to show iron loading by DAB-enhanced Perls' stain in any mice. Co-labeling with specific cell marker antibodies and DAB-enhanced Perls' staining revealed that iron was mostly accumulated in a subset of oligodendrocytes and some unidentified cells while neurons, astrocytes and microglia did not show clearly visible co-labeling with iron.

Gene expression microarray analysis revealed numerous transcriptome changes, including significant increases (fold change >1.28, $p<0.02$, n≥5) in brain transcripts for important immediate-early transcription regulators such as FBJ osteosarcoma oncogene (*Fos*), Jun B proto-oncogene (*Junb*), early growth response genes 1, 2 and 4 (*Egr1, 2* and *4*) and decreased transcripts for the transcription repressor *Zfp68*. These expression changes in key transcription factors are consistent with the numerous transcriptome changes observed and are likely to influence the downstream expression of multiple genes, affecting numerous

brain systems. Some of these changes appear likely to be compensatory responses while others may reflect perturbations of brain systems.

Several important genes related to iron metabolism showed decreased brain transcript levels, including the genes for transferrin (*Tf*), transferrin receptor 1 (*Tfr1*), ceruloplasmin (*Cp*) and hepcidin (*Hamp*). The decrease in *Tfr1* transcripts and high ferritin protein levels, described elsewhere (Heidari et al. 2015), are cellular responses typically seen with increased intracellular iron loading in other systems. These changes support increased iron storage in ferritin and reduced iron up take due to lower transferrin receptor expression. The reduction in transcripts for *Tf* and *Cp*, which encode two circulating extracellular proteins, may tend to decrease the amount of iron delivered from cerebral vascular endothelial cells into the brain and ultimately to neurons. All these changes are predicted to maintain brain iron homeostasis and help protect the brain against damage from high iron concentrations.

There were increased brain transcript levels for the hemoglobin alpha, adult chain 1 (*Hba-a1*) and both the hemoglobin beta, adult major and minor chains (*Hbb-b1, Hbb-b2*), possibly corresponding to neuronal hemoglobin expression, which may also have neuroprotective roles but could also cause tissue damage if released into the extracellular space.

Pathway analysis found significant alterations for a group of genes involved in the disease class Neurodegeneration with Brain Iron Accumulation (NBIA). These changes included increased level of ferritin protein (Heidari et al. 2015), reduced levels of transcripts for phospholipase A 2 group VI, fatty acid 2-hydroxylase, ceruloplasmin, chromosome 19 open reading frame 12 and ATPase type 13A2 (fold change >1.17, $p<0.04$, $n\geq5$ mice/group). Apart from the ferroxidase ceruloplasmin, all the other NBIA genes showing expression changes in response to iron were found to be either involved in myelin homeostasis or to contain mutations that result in demyelination in human patients or both. Further microarray data mining revealed that 18 other myelin-related genes also were down-regulated ($p<0.05$). This suggests that increased iron levels in the brain might exert effects on NBIA pathology through myelin related systems.

Brain transcriptome changes in the $Hfe^{-/-}xTfr2^{mut}$ mouse were then compared with expression profiles of normal human basal ganglia and brains from NBIA patients. Chi-

square testing showed significant overlap ($p<0.0001$) of differentially expressed genes in the *Hfe*$^{-/-}$*xTfr2*^{mut} brain with human brain gene networks that exhibited co-expression of NBIA and myelin-related genes. This suggests that increased iron loading selectively influences expression of a set of potentially interrelated NBIA and myelin genes.

There was also overlap ($p<0.0001$) of genes differentially expressed in the *Hfe*$^{-/-}$*xTfr2*^{mut} mouse brain and post-mortem NBIA basal ganglia. Pathway analysis showed that significant enrichment of 'myelin sheath' and various other myelin-related ontologies (all $p<0.05$). This suggests analogies between molecular mechanisms affected by brain iron loading in mice and pathogenic mechanisms in NBIA patients and provides further evidence for involvement of systems relating to myelin.

Collectively, these findings have confirmed the presence of higher brain iron content in the *Hfe*$^{-/-}$*xTfr2*^{mut} mice, as hypothesized, and provided evidence that this is primarily localized in myelinated structures, some oligodendrocytes and other still unidentified cells. The project also demonstrated using two experimental techniques (microarray, real time RT-PCR) that, as hypothesized, this increased brain iron loading was accompanied by altered expression of iron-related genes, that may protect the brain by increasing iron storage and decreasing iron uptake. Finally altered expression of NBIA genes related to myelin and other myelin-related genes, and correspondence between mouse transcriptome changes, human NBIA and myelin gene networks supports the hypothesis that the changes in iron lead to changes in gene expression relating to molecular systems involved in neurodegenerative disease. The results further provide evidence for the new hypotheses that myelin-associated iron accumulation may occur in hemochromatosis and that this may drives alterations in molecular systems involved in NBIA neuropathogenesis.

The research may shed light on potential interrelationships of iron and myelin in the brain in NBIA and other iron disorders, as well as in patients with psychiatric disorders or with myelin disorders such as multiple sclerosis.