**Iron accumulates in the brains of mice lacking the neurofilament light gene.**

James C. Vickers1, Anna E. King1, Graeme H. McCormack1, Aidan D. Bindoff1, Matthew T.K. Kirkcaldie1 and Paul A. Adlard2

1 Wicking Dementia Research and Education Centre, University of Tasmania

2 The Florey Institute of Neuroscience and Mental Health, University of Melbourne

Address for Correspondence:

James C. Vickers

Wicking Dementia Research and Education Centre

University of Tasmania

17 Liverpool St

Hobart

Tasmania 7000

Australia

e-mail: James.Vickers@utas.edu.au

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This study has determined that particular metal cations, such as iron, accumulate in the brains of animals that lack a neurofilamentous network in central neurons.



**Abstract**

There has been strong interest in the role of metals in neurodegeneration, and how ageing may predispose the brain to Alzheimer’s disease and Parkinson’s disease. Recent work has also highlighted a potential interaction between different metal species and various components of the cytoskeletal network in the brain, which themselves have a reported role in age-related degenerative disease and other neurological disorders. Neurofilaments are one such class of intermediate filament protein that have a demonstrated capacity to bind and utilise cation species. In this study, we investigated the consequences of altering the neurofilamentous network on metal ion homeostasis by examining neurofilament light (NFL) gene knockout mice, relative to wildtype control animals, at adulthood (5 months of age) and advanced age (22 months). Inductively coupled plasma mass spectroscopy demonstrated that the concentrations of iron (Fe), copper (Cu) and zinc (Zn) varied across brain regions and peripheral nerve samples. Zn and Fe showed statistically significant interactions between genotype and age, as well as between genotype and region, and Cu demonstrated a genotype and region interaction. The most substantial difference between genotypes was found in Fe in the older animals, where, across all regions examined, there was elevated Fe in the NFL knockout mice. This data indicates a potential relationship between the neurofilamentous cytoskeleton and the processing and/or storage of Fe through ageing.

**Significance to metallomics statement**

Neurofilament proteins have the ability to bind metal cations, and these metal species can assist in spacing between filaments as well the viscoelastic role of the neurofilamentous network. The major finding is that neurofilament deficiency in mice is associated with a marked increase in iron in the brain with ageing. In human brain, ageing-related changes in these cytoskeletal proteins may lead to excess iron, and contribute to susceptibility to neurodegenerative disease.

## Introduction

Divalent cation metal species such as zinc, iron and copper have important normal cellular functions in the nervous system, but may also be involved in pathological processes underlying neurodegenerative diseases such as Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease (AD) (Belaidi and Bush, 2016; Portbury and Adlard, 2017; Giampietro et al 2018). Indeed, metal ions have been implicated in many aspects of the AD cascade (Adlard and Bush, 2006), including a potentially central role in the aggregation of the two primary pathological hallmarks of AD, the beta amyloid plaques and neurofibrillary tangles (Cristóvão et al. 2016).

A range of brain proteins have the capacity to bind metal species, with major cell groups, such as neurons, microglia and astrocytes likely having a spectrum of roles in terms of the storage, metabolism and use of metals. Neurofilaments (NF) are major neuronal proteins and have putative roles in stabilising axons and regulating axonal diameter (Kirkcaldie and Dwyer, 2017). Particular subsets of neurons demonstrate high levels of the NF ‘triplet’ proteins (NF light (NFL), medium (NFM) and heavy (NFH)), and these correspond to subpopulations of neurons that are vulnerable to degeneration in AD (cortical pyramidal neurons), PD (substantia nigra compacta) and ALS (cortical, brainstem and spinal motor neurons) (Gai et al., 1994; Vickers et al., 2009, 2016). The NFM and NFH subunits have long tail domains that project from the intermediate filament backbone, and which contain glutamate-rich, negatively charged subregions. In this regard, these protein regions may bind to cations, with accessibility regulated by phosphorylation of the tail domains (Lefebvre and Mushynski, 1988).

Neurofilament accumulation in abnormal (dystrophic) neurites associated with beta amyloid plaques in the cortex is one of the earliest neuronal changes in the sequence of pathological changes leading to AD (Vickers et al., 2016). The NFL protein is a requisite component for the NF triplet to form intermediate filaments in neurons. We have also recently demonstrated that the ablation of NFL in a transgenic mouse model of AD (APPswe/PS1dE9) results in increased beta amyloid plaque deposition (Fernandez-Martos et al., 2015). These data suggest that the absence of NFs may have an early role in amyloid misprocessing. Furthermore, substantia nigra neurons in PD also lose NF immunolabelling (Gai et al., 1994), which is associated with the accumulation of iron in these nerve cells (Belaidi and Bush, 2016). Collectively, these data suggest that NF triplet-abundant neurons may show changes in these cytoskeletal proteins as part of the disease process of major neurodegenerative changes, and that such changes contribute to subsequent pathological alterations. In the following study, we have utilised mass spectrometry techniques to compare the abundance of metal species implicated in major neurodegenerative diseases, such as iron (Fe), copper (Cu) and zinc (Zn), in the brains and peripheral nerves of NFL KO mice as compared to C57BL/6 mice at adulthood (5 months of age) and following ageing (22 months of age).

## Methods

**Animals and tissue processing**

All animal experimentation was performed in accordance with the guidelines stipulated by the University of Tasmania Animal Ethics Committee (Approval Nos A12780 and A15120), which is in accordance with the Australian code of practice for the care and use of animals for scientific purposes. NFL KO mice were obtained from the Nathan Kline Institute (Dr. Mala Rao) and were developed in the laboratory of Dr. Jean-Pierre Julien (Zhu et al., 1997). NFL KO mice were maintained as a homozygous knockout colony. NFL KO mice were compared with wild-type (WT) (C57BL/6) mice, the predominant background strain of the mice (Liu et al., 2013; Zhu et al., 1997). Animals were housed in standard conditions (20ºC, 12/12 hours light/dark cycle), with at least 2 animals in each cage with access to food and water *ad libitum*. Cohort sizes were as follows, 5 months (n=12 NFL-KO, n=8 WT) and 22 months (n=7 NFL-KO, n=7 WT).

**Tissue collection and processing**

Animals were anaesthetised with 140mg/Kg (intraperitoneal) and perfused transcardially with phosphate buffered saline for 3 minutes to clear blood from the vasculature. Tissue was dissected under microscopic guidance. Briefly cortical tissue, hippocampus, olfactory bulb and cerebellum were collected from each hemisphere and the right hemisphere used in analysis; brain stem tissue was collected and left and right sciatic nerves were pooled for analysis. Upon collection, tissue was rapidly snap frozen in liquid nitrogen and stored at -80°C until used. Tissue was freeze dried in 96 well plates for 48hrs at -80˚C. Dried tissue was kept at room temperature until used.

**Inductively coupled plasma mass spectroscopy (ICPMS) analysis**

Tissue samples were coded and processed for inductively coupled mass spectroscopy analysis at the Florey Institute of Neuroscience and Mental Health. Tissue samples were lyophilised and then digested with nitric Acid (65% Suprapur, Merck) overnight, followed by heating at 90˚C for 20 min using a heat block. Samples were then removed from the heat block and an equivalent volume of hydrogen peroxide (30% Aristar, BDH) added to each sample. Once samples had finished digesting, they were heated for a further 15 mins at 70˚C. Samples were then diluted with 1% nitric acid diluent. Measurements were made using an Agilent 7700 series ICPMS instrument under routine multi-element operating conditions using a Helium Reaction Gas Cell.  The instrument was calibrated using 0, 5, 10, 50, 100 and 500 ppb of certified multi-element ICPMS standard calibration solutions (ICP-MS-CAL2-1, ICP-MS-CAL-3 and ICP-MS-CAL-4, Accustandard) for a range of elements, and we also utilised a certified internal standard solution containing 200 ppb of Yttrium (Y89) as a control (ICP-MS-IS-MIX1-1, Accustandard).

## Statistical Analysis

Mixed effects models were fitted using the lme4 package in R. Random intercepts for each subject were specified in order to account for non-independence between brain regions within animals. Model assumptions were checked using standard graphical techniques, and a -transformation applied to Zn and Fe variables to improve normality of residuals and homogeneity of error variance. One outlier was removed from Cu. Type III F statistics were computed using Kenward-Roger approximation, and 99% confidence intervals (CIs) were obtained by parametric bootstrapping. The choice of 99% CIs instead of 95% CIs reflects the exploratory and descriptive nature of the analysis, as there were no planned comparisons by which to determine a corrected for post-hoc testing. A measure of standardized effect size, Cohen's was calculated for the effect of genotype for each metal using the formula, where is the coefficient of determination for the full model, and is the coefficient of determination for a reduced model that does not contain the term of interest. The method of Nakagawa & Schielzeth (2012) for computing a marginal pseudo- for mixed models was used to calculate coefficients.

## Results and Discussion

Broadly, Fe content was increased in aged NFL KO and WT mice with ageing across most nervous system samples, with the exception of the olfactory bulb. In this regard, there was a more substantial increase in Fe content in NFL KO mice relative WT animals, particularly in the cerebellum, cortex, hippocampus and sciatic nerve (Figure 1). For many CNS regions, there was higher content of Zn in older NFL KO mice relative to WT mice. In addition, the sciatic nerve of NFL KO mice showed higher levels of Zn that WT mice at both 5 and 22 months of age. No consistent pattern of difference between NFL KO and WT mice was observed for Cu.

Genotype x age and genotype x region interactions were investigated for each metal cation in a model which accounted for region x age interactions and intra-class correlation within region for each animal. For Zn and Fe, there were significant genotype x age interactions (Zn: F(1, 27) = 9, *p* = .005; Fe: F(1, 27) = 11, *p* = .002) and genotype x region interactions (Zn: F(5, 140) = 8, *p* < .001; Fe: F(5, 140) = 3, *p* = .021). For Cu, only the genotype x region interaction was significant (F(5, 139) = 4, *p* = .002).

The standardized effect of genotype on Cu accumulation in tissues was small ( 0.09), however the standardized effect of genotype on Zn and Fe accumulation in tissues was moderate ( 0.27, 0.25).

The current study shows that there were differences in Cu, Zn and Fe concentrations across regions of the nervous system. For example, Fe was relatively abundant in the brain stem, with lower levels in the olfactory bulb. The olfactory bulb also showed relatively lower levels of both Zn and Cu. The sciatic nerve demonstrated very low levels of Cu, relative to Fe and Zn.

The most prominent differences between NFL KO and WT mice was found for Fe, which was accentuated with ageing. Moderate genotype differences were also detected for Zn, but these were not consistent across brain regions, although a pronounced difference was present for sciatic nerve. Consistent patterns of genotype difference for Cu were not observed. These studies were motivated by our previous observations that APPswe/PS1dE9 mice on a NFL KO background demonstrated higher amyloid plaque deposition (Fernandez-Martos et al., 2016). In this regard, we were interested in potential difference in brain content of Fe, Zn and Cu, metals implicated in beta amyloid processing and aggregation into plaques, that may be related to the absence of NFL and a complete neurofilamentous network (Zhu et al., 1997, Liu et al., 2013). The relatively increased levels of Fe across all brain regions of aged NFL KO mice may be particularly significant in the context of contributing to an environment that drives Alzheimer’s disease pathology. We have previously shown that there are substantial changes in NF localisation in the hippocampus during ageing, that abnormal accumulation of NFs in dystrophic neurites surrounding plaques is the earliest neuronal pathology of AD and that tau pathology replaces the normal NF network in neurofibrillary tangles and dystrophic neurites (Reviewed in Vickers et al., 2016). In this regard, changes to the integrity of neurofilamentous networks during ageing, which could possibly also follow decades of wear and tear on axons, may possibly result in increased levels of Fe in the extracellular environment, which may impact on multiple aspects of AD pathogenesis. The translation of the amyloid precursor protein (APP), which is the parent protein to the Aß which comprises the extracellular plaques in the AD brain, is regulated by an iron response element in the APP mRNA (Rogers et al., 1999, 2002) and the secretase cleavage of APP to form Aß is also regulated by iron. Furthermore, the aggregation and oligomerization of Aß is potentiated by iron, which is also likely to contribute to Aß-mediated oxidative damage (Huang et al., 1999 and 2004; Liu et al., 2011; Mantyh et al, 1993; Schubert et al., 1995; Jomova et al., 2010). Similarly, iron can induce the aggregation of tau (Yamamoto et al., 2002) and the association of redox active iron with tau and neurofibrillary tangles may contribute to oxidative stress (Good et al., 1992). In addition, a loss of NFs in substantia nigra neurons in the early stages of PD (Gai et al. 1994) may also be linked to the accumulation of Fe in these cells (Belaidi and Bush, 2016).

The current data supports the existence of a relationship between NF protein levels and metal ions in the brain, potentially relating to the regulation and/or storage of metals. This is supported by literature demonstrating that cations may also have direct roles in the organisation of neurofilamentous networks. Iron deficiency, for example, can impact neurofilament phosphorylation during development (Lee et al 2012). Copper and iron may also have a role in catechol-mediated cross-linking of neurofilament proteins (Montine et al. 1995), and copper and zinc have the capacity to directly bind to the NFH subunit (Pierson and Evenson, 1988). Divalent ions such as Ca, Mg and Zn have been shown to act as cross-linkers between NFs, influencing the gelation and viscoelastic properties of neurofilamentous networks (Yao et al., 2010; Pregent et al. 2015). Finally, atomic force microscopy experiments support the cross-linking role of divalent cations, particularly in moderating the spacing and mechanical properties of phosphorylated NF side-arm structures (Lei et al., 2018).

## Conclusion

Cumulatively, these data show that the absence of NF-L, and a neurofilamentous network perturbs the processing and/or storage of Fe through ageing, leading to an accumulation of this metal in the brain and sciatic nerve. Alterations in neurofilaments during brain ageing may, thus, create an intracellular or extracellular environment of excess Fe, which could contribute to risk of either neuronal degeneration or protein accumulation in neurodegenerative conditions such as Alzheimer’s and/or Parkinson’s disease.

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Figure 1. Fe, Zn, and Cu in sampled brain regions at 5mo and 22mo, for WT and NFL KO mice. Error-bars show mean and 99% CI, revealing the strength and direction of these patterns of metal accumulation. For transparency, data are shown as points.