Redox stress and metal dys-homeostasis appear as hallmarks of early prion disease pathogenesis in mice

Jereme G Spiers[1#†], Hsiao-Jou Cortina Chen[2†], Tiffany Barry[3], Julie-Myrtille Bourgognon[4], Joern R Steinert[5*]

† Contributed equally to this work

***Correspondence to:**

* Joern.Steinert@nottingham.ac.uk

j.spiers@latrobe.edu.au

Addresses:

1, JGS: Department of Biochemistry and Chemistry, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Victoria, 3083, Australia

2, HJCC: Metabolic Research Laboratories, Wellcome Trust MRC Institute of Metabolic Science, University of Cambridge, Addenbrooke's Hospital, Cambridge, CB2 0QQ, United Kingdom

3, TB: School of Geography, Geology and the Environment,
University of Leicester, University Road, Leicester, LE1 7RH, UK

4, J-MB: Institute of Infection, Immunology and Inflammation, Sir Graeme Davies Building, 120 University Place, Glasgow G12 8TA, UK

5, JRS: Division of Physiology, Pharmacology and Neuroscience
University of Nottingham, School of Life Sciences, Nottingham, NG7 2NR, UK

Abstract

Neurodegenerative diseases are associated with a multitude of dysfunctional cellular pathways. One major contributory factor is a redox stress challenge during the development of several protein misfolding conditions including Alzheimer's (AD), Parkinson's disease (PD), and less common conditions such as Creutzfeldt Jakob disease (CJD). CJD is caused by misfolding of the neuronal prion protein and is characterised by a neurotoxic unfolded protein response involving chronic endoplasmic reticulum stress, reduced protein translation and spongiosis leading subsequently to synaptic and neuronal loss. Here we have characterised prion disease in mice to assess redox stress components including nitrergic and oxidative markers associated with neuroinflammatory activation. Aberrant regulation of the homeostasis of several redox metals contributes to the overall cellular redox stress and we have identified altered levels of iron, copper, zinc, and manganese in the hippocampus of prion diseased mice. Our data show that early in disease, there is evidence for oxidative stress in conjunction with reduced antioxidant superoxide dismutase mRNA and protein expression. Moreover, expression of divalent metal transporter proteins was reduced for *Atp7b*, *Atox1*, *Slc11a2*, *Slc39a14* (at 6-7 weeks) but increased for *Slc39a14* and *Mt1* at 10 weeks of disease. Our data present evidence for a strong pro-oxidant environment and altered redox metal homeostasis in early disease pathology which both maybe contributory factors to aggravating this protein misfolding disease.

Keywords: Essential redox metals; hippocampus; nitrosative stress; oxidative stress; prion

Abbreviations:

4-hydroxynonenal (4-HNE)

Antioxidant 1 copper chaperone (Atox1)

Amyloid β (Aβ)

Alzheimer's (AD)

Creutzfeldt Jakob disease (CJD)

Cellular prion (PrP^{C})

Copper chaperone for superoxide dismutase 1 (Ccs)

Divalent metal transporter 1 (DMT1)

Gerstmann-Sträussler-Scheinker disease (GSS)

Inducible nitric oxide synthase (iNOS)

Nitric oxide (NO)

Normal brain homogenate (NBH)

Parkinson's disease (PD)

Scrapie prion protein (PrP^{Sc})

Super oxide dismutase (SOD)

Rocky Mountain Laboratory scrapie prion (RML)

Transmissible spongiform encephalopathies (TSEs)

Weeks post-inoculation (w.p.i.)

ZRT/IRT-like protein 14 (ZIP14)

Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are progressive and fatal neurodegenerative conditions associated with accumulation and propagation of the scrapie prion protein (PrP^{Sc}) [1, 2] resulting in conditions such as Creutzfeldt Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker disease (GSS) in human or bovine spongiform encephalopathy (BSE) in cow and Scrapie in sheep and goats. The molecular mechanisms responsible for prion-induced neurodegeneration remain to be elucidated but involve multiple processes operating synergistically and simultaneously, and centre around the loss of cellular prion (PrP^{C}) function and accumulation of the cytotoxic misfolded scrapie form of the protein (PrP^{Sc}). These processes include the development of spongiform lesions, increased neuroinflammation, oxidative stress, and neuronal/glial cell dys-function [3-5]. The most typical brain alteration in prion diseases is vacuolation and previous studies have demonstrated that depleting endogenous neuronal prion protein reversed the spongiform change in mice with early prion infection [6]. Recently, Lakkaraju and colleagues (2021) identified a key component of prion-induced spongiosis involves chronic endoplasmic reticulum stress and the downstream deacylation and destabilisation of an inositide kinase, PIKfyve, resulting in its depletion in prion infected mouse and human brain [7]. Reportedly, endoplasmic reticulum stress also induces a suppression of translational pathways, which could be reversed by overexpression of GADD34, a specific eIF2α-P phosphatase thereby restoring translation and reducing neuronal loss [8]. Furthermore, it is clear that prion diseases involve widespread glial activation and numerous proinflammatory effectors have been shown to increase in the brain of prion diseased animal models and patients with TSEs [9-11]. Hwang and colleagues (2009) have demonstrated that genes associated with microglial proliferation and astrocytosis are differentially expressed following prion infection regardless of prion strain or mouse genetic background [12]. This was evident well before

any clinical signs of disease or neuronal damage which suggests that brain inflammation might be a direct response to the progressive deposition of PrP^{Sc}. More specifically in the hippocampus, a recent study using genome-wide transcriptomics identified the appearance of clinical symptoms coincided with early microglia alterations and that microglial activation drives symptomatic prion disease progression in mice inoculated with RML6 prions (passage 6 of Rocky Mountain Laboratory strain mouse-adapted scrapie prions) [13]. In agreement with these studies, we have previously demonstrated increased neuroinflammatory and altered nitric oxide signalling with an accompanied nitrergic stress in the hippocampus of mice 9 weeks post-inoculation (w.p.i.) with scrapie prion protein [9, 14]. Pharmacological *in vivo* suppression of nitric oxide production showed beneficial effects on hippocampal physiology and supressed prion protein aggregation [9] illustrating the importance of nitrergic stress in this protein misfolding neuropathology. In addition to the enhanced neuroinflammatory signalling and the resultant nitrergic stress, oxidative stress and damage are the other hallmarks of pathology observed in protein misfolding diseases including prion disease. It has been demonstrated previously that the PrP^{C} can act as an antioxidant, while the PrP(106-126) fragment which corresponds to the neurotoxic PrP^{Sc} increases reactive oxygen species generation and mitochondrial dysfunction *in vitro* [15-17]. Scrapie prion-infected or PrP(106-126)-treated neuronal cultures also induced a significant decrease in antioxidative activity including superoxide dismutase and glutathione dependent antioxidant systems [18, 19]. Together, the generation of oxidants and altered redox balance can cause impairment of mitochondrial activity and turnover which has been reported widely in humans and animal models of prion diseases [20-22]. Furthermore, oxidative stress-induced lipid peroxidation and the consequent generation and accumulation of the highly electrophilic aldehydes, 4-hydroxynonenal (4-HNE), indicative of oxidative damage have been observed in brains of prion infected mice and human diseased tissues [23].

There are several lines of evidence which describe PrP^C as an important metal binding protein for divalent metals including manganese (Mn), and zinc (Zn), in addition to redox-sensitive metals, such copper (Cu) and iron (Fe) [24-27]. However, in the state of prion disease, the loss of PrP^C function may result in metal dys-homeostasis and contribute to the exacerbation of oxidative stress and damage [28, 29]. Using the ME7 and 139A mouse adapted scrapie strains, Wong and colleagues (2008) have demonstrated that divalent metal contents were significantly perturbed following three weeks of prion infection and these correlated with a reduced enzymatic activity of antioxidative copper/zinc superoxide dismutase [30]. Furthermore, Canello and colleagues (2012) have shown that long term oxidative insult through administration of copper significantly accelerated the onset and progression of spontaneous prion disease in TgMHu2ME199K mice [31]. In the same study, using fibroblasts from TgMHu2ME199K and PrP^C-ablated mice, they demonstrated that a functional PrP^C is necessary to protect cells against copper toxicity. Under normal physiological conditions, the metabolism and intracellular concentration of metal ions is strictly regulated by metal transporters which mediate the import, distribution, and export from cells. For example, the uptake of copper into neurons is primarily regulated by copper transporter 1. The intracellular copper transfer is facilitated by specific copper chaperones such as antioxidant 1 copper chaperone (Atox1) and copper chaperone for superoxide dismutase 1 (Ccs). The divalent metal transporter 1 (DMT1) is a multi-metal transporter with a primary role in iron trafficking while ZRT/IRT-like protein 14 (ZIP14) is a transport protein that can mediate cellular uptake of iron, zinc and manganese. Interestingly, Tripathi and colleagues (2015) have reported that PrP^C in the liver can function as a ferrireductase partner for both DMT1 and ZIP14 which, during prion disease pathogenesis, is likely to alter the homeostasis of most key essential metals [32]. In this study, we elucidated the alterations in prion-induced metal homeostasis in the hippocampus through expression analysis of different

metal transporters at an early and late stage of prion disease progression in mice and correlate these data with information detailing the hippocampal redox state.

Materials and Methods

All animal work was performed under the listed procedures detailed in Project Licence PPL 70/8808 and conformed to the UK Home Office regulations and institutional guidelines. The procedures for prion inoculation have been well characterised and outlined previously [8]. Briefly, at 3 weeks of age, hemizygous Tg37 mice expressing approximately 3-4 times the normal PrP^C were inoculated with either Rocky Mountain Laboratory scrapie prion (RML) or normal brain homogenate (NBH). Inoculated mice were allocated into groups of n=2-4 for biochemical, molecular or immunohistochemical analysis. Mice were sacrificed at 6-7 and 10 w.p.i. with the hippocampus dissected and either flash frozen in liquid nitrogen for storage at -80^{o}C or fixed with 4% paraformaldehyde. The two time-points represents preclinical asymptomatic and subclinical stages of prion disease.

Hippocampal iNOS ELISA

Isolated hippocampal tissues were homogenised in RIPA buffer containing protease inhibitors (Roche), centrifuged (12,000 x g, 4^{o}C, 15 min), and the supernatant was collected for biochemical analysis. Protein levels were determined using a commercially available Bradford protein assay (ab102535; Abcam) and equal quantities of protein were used for inducible nitric oxide synthase (iNOS) assays. Hippocampal iNOS (ab253219; Abcam) were determined using commercially available ELISA according to the manufacturer's instructions.

Inductively coupled plasma mass spectrometry (ICP-MS)

All experiments were performed using a Thermo Scientific iCAP-Qc quadrupole ICP mass spectrometer. Isolated hippocampal samples were prepared according to methods outlined in Hare & Colleagues (2009) with modifications [33]. Briefly, weighed hippocampal tissues were initially homogenised in 18.2 mΩ deionised H_2O and volumes containing equivalent tissue were transferred to clean tubes. Matrix-matched tissue standards were constructed using chicken breast tissue spiked with appropriate concentrations of Fe, Cu, Zn, and Mn. Standards and samples were subsequently digested in a 3:1 mixture of 70% HNO_3 and 36% HCl using a microwave digester. Digests were diluted to 50 g in 18.2 mΩ deionised H_2O and a 10 mL aliquot was removed to a new tube and spiked with an Rb/Ir mixture as internal reference elements. Sample values for each element were corrected for tissue wet weight and compared to the NBH group.

Immunocytochemistry

Isolated tissues were post-fixed with 4% paraformaldehyde, cryoprotected with graded sucrose, and frozen in Optimal Cutting Temperature solution for sectioning and immunofluorescent analysis. Embedded tissues were cryo-sectioned at 12 µm and thaw-mounted onto Superfrost Plus slides, dried, and stored at -20°C. Immunofluorescent staining was performed by washing sections three times in PBS containing 0.1% Triton X-100 (PBS-T) followed by antigen retrieval in citrate buffer at 95°C. Sections were washed and blocked in PBS-T containing 10% normal goat serum and 1% BSA for 1 hour at room temperature followed by overnight incubation in primary antibodies (iNOS: Ab49999 – Abcam; 4-HNE: MA5-27570 – ThermoFisher Scientific; SOD1: ADI-SOD-100 – Enzo Life Sciences; NeuN:

Ab104225 – Abcam; NeuN: Mab377 – Millipore; Tyrosine Hydroxylase: Ab152 - Millipore). Sections were washed in PBS-T and incubated in fluorescent secondary antibodies (Goat anti-mouse Alexa Fluor 488 – Cat # A11001; Goat anti-rabbit Alexa Fluor 594 – Cat #32740) for 2 hours at room temperature followed by washing and nuclear staining with DAPI prior to mounting in Prolong gold antifade mounting media (P36930 – ThermoFisher Scientific). Sections of the dorsal hippocampus were imaged using a Zeiss LSM 510 META NLO confocal microscope and captured using Zen 2009 software (Zeiss). Furthermore, representative images of the locus coeruleus were captured using positive tyrosine hydroxylase staining to identify locus coeruleus boundaries for further immunofluorescent image analysis.

mRNA Expression

Total RNA was extracted from hippocampal tissue using a RNeasy mini kit which included an on-column deoxyribonuclease 1 treatment step. A total of 1 µg RNA was reverse transcribed using the iScript cDNA synthesis kit and real-time PCR was performed with 25-100 ng of cDNA per reaction. Target genes of interest were determined using the Taqman gene expression 'assay on demand' assays and the primer/probe targets were FAM-labelled Atox 1 (Mm00839626_m1), Atp7b (Mm00599675_m1), Ccs (Mm00444148_m1), Mt1 (Mm00496660_g1), Slc11a2 (Mm00435363_m1), Slc39a14 (Mm01317439_m1), Mpo (Mm01298424_m1), Sod1 (Mm01700393_g1), and Sod2 (Mm01313000_m1). Expression of target genes were normalised using a multiplexed reaction with a VIC-labelled β-actin (Actb; Mm00607939_s1) and relative expression was determined using the $\Delta\Delta^{-CT}$ method using 6 w.p.i. NBH-treated mice as controls.

Statistical analysis

All data in this study was analysed using GraphPad Prism 8.0.1 (GraphPad Software, Inc.). One-way and Two-way ANOVA were performed followed by Fisher's Least Significant Difference (LSD) test to identify statistically significant differences between experimental groups at 6-7 and 10 w.p.i. timepoints. Unpaired t-test was used to compare significant differences at the subclinical stages of prion disease (10 w.p.i.). Results were presented as mean ± standard error of the mean (± SEM) and p-values less than 0.05 were considered as statistically significant.

Results

Upregulation of inducible nitric oxide synthase occurs early in prion disease

Neuroinflammation is a common feature of several neurodegenerative diseases and is strongly associated with microglial activation and the proinflammatory upregulation of iNOS. The hippocampal protein expression of iNOS was significantly elevated in RML-inoculated mice [$F_{(2, 7)} = 5.170$, $p=0.0418$] at 10 w.p.i. ($p<0.05$) when compared to the NBH group (Fig. 1A). This is supported by immunohistochemical analysis of iNOS which further demonstrated iNOS expression was found in non-neuronal cells of the hippocampus (Fig. 1B). Quantification of hippocampal iNOS-positive cells per field demonstrated a significant treatment effect [$F_{(1, 5)}=28.64$, $p=0.0031$] with Fisher's post-test analysis indicating higher iNOS-positive cells per fields in the RML group at both 6-7 w.p.i. ($p<0.01$) and 10 w.p.i. ($p<0.05$) (Fig. 1C). Early changes in iNOS were also detected in the locus coeruleus, a region known to be susceptible to early prion protein aggregation in prion disease [34] (Supplementary Fig S1A & C).

Oxidative stress appears early in prion disease pathogenesis

Neuroinflammation also disrupts redox balance, typically resulting in oxidative stress which is characterised by increased production and accumulation of free radicals and reduced antioxidant defence. The reactive aldehyde, 4-HNE, is a major bioactive marker of lipid peroxidation and with the high lipid content in neural tissue, this makes 4-HNE a sensitive marker of oxidative stress in the brain. Two-way ANOVA have demonstrated significant effects of prion inoculation [$F_{(1, 5)}$=45.77, p=0.0011] and the interaction between the prion treatment and time [$F_{(1, 5)}$=6.962, p=0.0461] on 4-HNE immunoreactivity. The RML-induced increase in 4-HNE immunoreactivity was significant at the early 6-7 w.p.i. ($p<0.05$) and persisted to the 10 w.p.i. ($p<0.001$) timepoint (Fig. 2A & 2B). Similarly, increased 4-HNE immunoreactivity was also observed in the locus coeruleus of RML prion inoculated mice compared to NBH controls (Supplementary Fig. S1B & D).

We have further demonstrated a significant prion-induced [$F_{(1, 5)}$=12.01, p=0.0179] decrease in the antioxidative SOD1 immunoreactivity in the hippocampus. Fisher's post-test indicated lower SOD1 signal following 10 w.p.i. ($p<0.05$) in the RML-inoculated group compared to matched NBH controls (Fig. 3A & 3B). This is observed alongside a decrease in the mRNA expression of Sod1 in the hippocampus of prion-diseased [$F_{(1, 12)}$=50.92, $p<0.0001$] mice at both the 6-7 ($p<0.001$) and 10 ($p<0.01$) w.p.i. (Fig. 4A). The mRNA expression of manganese-dependent superoxide dismutase (Sod2) was also downregulated in the RML-infected [$F_{(1, 12)}$=15.51, p=0.002] hippocampus following 10 w.p.i. ($p<0.01$) (Fig. 4B). Next, we examined the mRNA expression of a major copper chaperone, Ccs, which is responsible for copper incorporation to SOD1 for its catalytic activity. Figure 4C revealed that hippocampal Ccs mRNA expression was significantly downregulated in prion-diseased mice [$F_{(1, 12)}$=51.75, $p<0.0001$] in a time-dependent manner [$F_{(1, 12)}$=6.361, p=0.0268] at both 6-7 ($p<0.001$) and 10 ($p<0.01$) w.p.i. when compared to the NBH-group. The interaction between

these two factors was also significant [$F_{(1, 12)}$=7.703, p=0.0168] which may suggest that the dismutase activity of SOD1 can be affected by alterations in copper homeostasis following prion inoculation. Together, with the increased 4-HNE and decreased overall mRNA and protein expressions of superoxide dismutase, we have shown that hippocampal oxidative stress is observable at an early time-point of prion disease which was further exacerbated at the later stage of the disease.

Metal dys-homeostasis coincides with altered expression of metal transporters and binding proteins

The observed reductions in Sod1 and Ccs led us to examine redox active metals in prion diseased hippocampal tissues. The normal prion protein is strongly associated with copper to which it binds with high affinity, and therefore we have measured the levels of two naturally occurring isotopes, ^{63}Cu and ^{65}Cu. Here, we observed a time-dependent effect in both ^{63}Cu [$F_{(1, 10)}$=15.13, p=0.003] and ^{65}Cu [$F_{(1, 10)}$=15.94, p=0.0025] which both elevated significantly in the RML-inoculated group at 10 w.p.i. when compared to the early timepoint of 6 w.p.i. ($p<0.05$) (Fig. 5A &5B). However, no significant difference was found between NBH and RML at either 6 and 10 w.p.i. for both ^{63}Cu and ^{65}Cu. Upon entry to the cell, copper transfer is facilitated by Atox1 and copper chaperone for Sod1 (Ccs) as shown above. Figure 5C shows that RML prion [$F_{(1, 12)}$=8.248, p=0.014] significantly reduced the mRNA expression of Atox1 when compared to the NBH- group at 6 w.p.i. ($p<0.01$) in the hippocampus. Copper delivery to the secretory pathway is further facilitated by the membrane-associated copper-transporting ATPases including Atp7b. We have demonstrated in Figure 5D that prion [$F_{(1, 12)}$=14.79, p=0.0023] induces a sustained suppression of Atp7b mRNA levels at both time-points examined ($p<0.01$).

Next we examined tissue levels of zinc and specifically two of the stable isotopes of ^{66}Zn and ^{68}Zn. Two-way ANOVA revealed that there is a significant main effect of RML-prion [$F_{(1, 11)}$=8.337, p=0.0148] and time [$F_{(1, 11)}$=28.44, p=0.0002] and the interaction between these two factors [$F_{(1, 11)}$=16.28, p=0.002] on hippocampal ^{66}Zn, with elevated levels appearing at 10 w.p.i. (p<0.001) (Fig. 6A). Similarly, ^{68}Zn also demonstrated significant prion-induced [$F_{(1, 11)}$=9.55, p=0.0103] and time-dependent [$F_{(1, 11)}$=29.0, p=0.0002] effects and the interaction between treatment and time [$F_{(1, 11)}$=20.12, p=0.0009], with post hoc analysis showing prion-induced increases when compared to the NBH controls following 10 w.p.i. (p<0.001) (Fig. 6B). We also examined the mRNA expressions of two major zinc-binding and transport proteins including Slc39a14 (Zip14) and Mt1 in the brain. Figure 6C demonstrates that the effects of time [$F_{(1, 12)}$=8.068, p=0.0149] and the interaction between time and treatment [$F_{(1, 12)}$=34.70, p<0.0001] is significant at inducing the observed decrease in Slc39a14 mRNA expression at 6 w.p.i. (p<0.001) which becomes upregulated following 10 w.p.i. (p<0.01) in the RML-infected hippocampus. For hippocampal Mt1, two-way ANOVA showed that there is a significant effect of prion inoculation [$F_{(1, 12)}$=20.16, p=0.0007] and time [$F_{(1, 12)}$=23.07, p=0.0004] and the interaction between these two main factors [$F_{(1, 12)}$=25.73, p=0.0003]. Fisher's post-test further revealed a strong upregulation in Mt1 mRNA at 10 w.p.i. (p<0.001) in the RML prion group when compared to NBH controls (Fig. 6D).

Lastly, we assessed the effects of RML-prion on hippocampal iron (^{54}Fe and ^{56}Fe) and manganese ($_{55}$Mn). Figure 7A shows a significant time-dependent effect [$F_{(1, 11)}$=13.19, p=0.0039] and the interaction between time and prion treatment [$F_{(1, 11)}$=21.27, p=0.0007] on

^{54}Fe, with significant increases at 6 ($p<0.05$) and 10 ($p<0.01$) w.p.i. in the hippocampus. A significant effect of time [$F_{(1, 11)}=12.72$, $p=0.0044$] and the interaction between time and prion inoculation [$F_{(1, 11)}=15.75$, $p=0.0022$] was detected for hippocampal ^{56}Fe, becoming significantly increased at 10 w.p.i. ($p<0.01$) (Fig. 7B). For ^{55}Mn, there was a significant time-dependent effect [$F(1, 11)=23.53$, $p=0.0005$] and the interaction between time and treatment [$F(1, 11)=20.12$, $p=0.0009$], with post-test analysis revealing an initial decrease at 6 w.p.i. ($p<0.05$) and subsequent increase at 10 w.p.i. ($p<0.01$) (Fig. 7C). Finally, we have examined the mRNA expression of Slc11a2 which encodes a divalent metal transporter (DMT1) and carries iron, manganese, copper, zinc, and other major essential metals in the hippocampus. Two-way ANOVA demonstrated that there is a significant main effect of RML-prion [$F(1, 12)=28.28$, $p=0.0002$] on hippocampal Slc11a2 mRNA expression which was downregulated at both 6 and 10 w.p.i. ($p<0.01$) when compared to the respective NBH-groups (Fig. 7D).

Discussion

Neurodegenerative disorders are characterised by a complex network of pathological events among which protein misfolding, neuroinflammation, oxidative stress, mitochondrial dysfunction, redox metal ions dys-homeostasis, metabolic changes, and apoptotic neuronal death have been widely described [14, 35-37]. One such response to protein misfolding results in upregulation of neuroinflammatory pathways connecting enhanced redox stress to aberrant nitrergic activity. Furthermore, dysregulation of redox metal homeostasis has emerged as an important factor in contributing to pathologies in neurodegeneration. In addition, it is not well established to what extend the presence of the accumulating misfolded proteins, such as prion, amyloid β (Aβ), α-synuclein or huntingtin versus the absence of function of the native cellular proteins determines the cell fate. Many studies in different

animal models have provided compelling evidence that the presence of misfolding proteins is sufficient to cause disease phenotypes but also elucidated the functions of these cellular proteins required for physiological processes [38-43].

Our findings show that the early pathology of prion disease is associated with enhanced redox stress due to increased amounts oxidative and nitrergic signalling as confirmed by increased expression of iNOS, 4-HNE staining and a reduced antioxidant capacity as detected by lower SOD-1/2 protein and gene expressions (Figs. 1-3). Moreover, these changes were found in both the hippocampus and locus coeruleus, regions known to be susceptible to prion protein aggregation during the pathogenesis of prion disease [34]. This is in agreement with our previous work demonstrating increased nitrosative/oxidative stress in prion diseased mice [9, 14]. Furthermore, we found that the levels of several important redox metals are dysregulated in prion disease possibly due to changes in expression levels of divalent metal transporters. Many of these changes occur during the early phases of the pathology at around 6-7 w.p.i. prior to detectable synaptic or neuronal dysfunction [9] suggesting that they potentially contribute to the progression of disease. Elevated iron levels are associated with increased oxidative stress which, via the Fenton Reaction, mediates further lipid peroxidation. This lipid peroxidation of membrane bound polyunsaturated fatty acids via iron-induced free radicals, with concomitant deficiency in glutathione-mediated antioxidant defence, provides a target for treatment to reduce ferroptosis and associated neurotoxicity [44]. Other studies have reported increased iron levels in the central nervous system in AD [45, 46] which can facilitate aggregation of Aβ *in vitro* [47] in mouse models of AD [48] and in the substantia nigra pars compacta in PD [49]. Increased iron levels are also detected in microglia, astrocytes, and dopaminergic neurons in PD [46, 50] illustrating the broad involvement of iron dysregulation in neurodegeneration (see review [51]). It has been reported that nitric oxide-mediated S-nitrosylation of the DMT1 leads to an increase in cytosolic iron [52]. This

could potentially explain our data showing higher hippocampal iron and manganese levels despite the fact that the gene expression for *Slc11a2* is reduced (Fig. 6A, B).

Dysregulation of other redox metals has equally been reported in neurodegenerative conditions. Both elevated and reduced levels of zinc have been shown in AD [53, 54]. Intracellular excess of zinc, being a consequence of Aβ aggregation and elevated redox stress, releases more zinc from metallothioneins which may affect mitochondrial function and induce apoptosis.

Accumulation of various redox metals has been associated with increased levels of their transports, with a recent study targeting these metal transporters demonstrating therapeutic potential in diseases involving prion-like polymers including PD [55]. As copper not only interacts with the prion protein, an imbalance is strongly associated with neurodegeneration in dementia, but a complete biochemical characterisation consistent with the clinical and genetic data is required to support a causative association, rather than just correlation with disease. Our data show that the copper ATPase Atp7b is highly downregulated early in pathology (Fig. 4). Dysfunction of this transporter is also associated with Wilson Disease [56] and motor neuron disease [57] linking degeneration to a copper dys-homeostasis.

In summary, our data present evidence for an enhanced redox stress early in prion disease. This condition is defined by an elevated oxidative and nitrergic stress environment, compounded by a reduced antioxidant capacity. We further showed that an imbalance of redox metals occurs prior to detectable neuronal and synaptic dysfunction potentially causing further neuronal damage. This metal dys-homeostasis may be related or aggravated by changes in function and expression of divalent metal transporter proteins which were observed during early disease pathogenesis.

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Conflict of Interests

The authors declare no conflict of interest.

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Figure Legends

Figure 1: Hippocampal iNOS upregulation contributes to the early pathogenesis of prion disease

Expression of hippocampal iNOS protein (A) was increased in RML prion mice 10 w.p.i. when compared to mice inoculated with NBH (n=3-4 per group). Representative images (B) and image quantifications(C) at 6 & 10 w.p.i show significant increases in hippocampal

iNOS immunoreactivity (green) in RML prion mice were not observed in neuronal cells stained with NeuN (red)(n=2-3 per group). Data are expressed as mean ± SEM, $^{*}p < 0.05$ and $^{**}p < 0.01$.

Figure 2: Enhanced hippocampal oxidative stress is a feature of early prion disease

Representative images (A) and image quantifications (B) at 6 & 10 w.p.i. show increased hippocampal 4-HNE immunoreactivity (green) in RML prion mice were heavily localised to neuronal cells stained with NeuN (red)(n=2-3 per group). Data are expressed as mean ± SEM, $^{*}p < 0.05$ and $^{***}p < 0.001$.

Figure 3: Reduced hippocampal SOD1 immunoreactivity occurs following RML prion inoculation

Representative images (A) and image quantifications (B) at 6 & 10 w.p.i. show hippocampal SOD1 immunoreactivity (green) in neuronal cells stained with NeuN (red) in RML prion mice was found to be significantly reduced at 10 w.p.i. (n=2-3/group). Data are expressed as mean ± SEM, $^{*}p < 0.05$.

Figure 4: Expression of SOD1/2 and the copper chaperone for superoxide dismutase mRNA is reduced in prion disease

There was reduced expression of Sod1 (A) at both 6-7 & 10 w.p.i while reductions in Sod2 (B) expression were only observed at 10 w.p.i. in RML prion mice when compared to age-matched NBH control mice. The copper chaperone for superoxide dismutase, Ccs, showed strong reductions in expression at both 6-7 & 10 w.p.i in RML prion mice compared to NBH

control mice (C)(n=4 per group). Data are expressed as mean ± SEM, **p < 0.01 and ***p < 0.001.

Figure 5: Hippocampal copper homeostasis shows subtle changes in prion disease

There were no significant changes in hippocampal ^{63}Cu or ^{65}Cu ions following prion inoculation (A & B)(n=3-4 per group). However, there were subtle changes in expression of the copper metallochaperone, Atox1 (C), which showed reduced expression at 6-7 w.p.i. in RML prion inoculated mice. The copper transporter, Atp7b, also displayed subtle reductions in expression at both 6-7 & 10 w.p.i. in RML prion mice when compared to the NBH control mice (D)(n=4 per group). Data are expressed as mean ± SEM, *p < 0.05 and **p < 0.01.

Figure 6: Hippocampal zinc show strong changes particularly in later prion disease pathogenesis

Hippocampal zinc ions ^{66}Zn and ^{68}Zn both displayed robust increases in RML prion inoculated mice later at 10 w.p.i. when compared to NBH control animals (A & B)(n=3-4 per group). The divalent metal transporter, Slc39a14, was downregulated initially at 6-7 and subsequently upregulated later in disease at 10 w.p.i. in RM L prion mice compared to NBH controls (C). The zinc binding protein, Mt1, was strongly upregulated later in prion disease at 10 w.p.i. in RML prion mice compared to NBH controls (n=4 per group). Data are expressed as mean ± SEM, **p < 0.01 and ***p < 0.001.

Figure 7: Divalent metals and transporter show subtle changes predominately in later prion disease pathogenesis.

Hippocampal iron 54Fe displayed subtle reductions in hippocampal tissue at 6-7 w.p.i., with both 54Fe and 56Fe showing stronger increases occurring later at 10 w.p.i. in RML prion mice compared to NBH controls (A & B)(n=3-4 per group). Hippocampal manganese 55Mn was also increased later at 10 w.p.i. in RML prion inoculated mice (C). Expression of the divalent metal transporter, Slc11a2, showed early and sustained reductions at 6-7 & 10 w.p.i. in RML prion mice compared to NBH controls (n=4 per group). Data are expressed as mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

Figure S1: Increased nitrosative/oxidative markers are found in the locus coeruleus during the early development of prion disease.

Representative images (A) and image quantifications (B) at 6 & 10 w.p.i. show locus coeruleus iNOS increases in RML prion-inoculated mice compared to NBH controls (n=3-4 per group). Representative images (C) and image quantifications (D) at 6 & 10 w.p.i. show locus coeruleus 4-HNE increases in RML prion-inoculated mice compared to NBH controls (n=3-4 per group). Data are expressed as mean ± SEM, *$p < 0.05$ and **$p < 0.01$.