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OXIDATIVE INJURY IN MULTIPLE SCLEROSIS CEREBELLAR GREY MATTER

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Abstract

Cerebellar dysfunction is a significant contributor to disability in multiple sclerosis (MS). Both white matter (WM) and grey matter (GM) injury occurs within MS cerebellum and, within GM, demyelination, inflammatory cell infiltration and neuronal injury contribute to on-going pathology. The precise nature of cerebellar GM injury is, however, unknown. Oxidative stress pathways with ultimate lipid peroxidation and cell membrane injury occur extensively in MS and the purpose of this study was to investigate these processes in MS cerebellar GM. Post-mortem human cerebellar GM from MS and control subjects was analysed immunohistochemically, followed by semi-quantitative analysis of markers of cellular injury, lipid peroxidation and anti-oxidant enzyme expression. We have shown evidence for reduction in myelin and neuronal markers in MS GM, coupled to an increase in expression of a microglial marker. We also show that the lipid peroxidation product 4-hydroxynonenal co-localises with myelin and its levels negatively correlate to myelin basic protein levels. Furthermore, superoxide dismutase (SOD1 and 2) enzymes, localised within cerebellar neurons, are up-regulated, yet the activation of subsequent enzymes responsible for the detoxification of hydrogen peroxide, catalase and glutathione peroxidase are relatively deficient. These studies provide evidence for oxidative injury in MS cerebellar GM and further help define disease mechanisms within the MS brain.

Key Words

Multiple sclerosis; cerebellum; grey matter; oxidative stress; peroxidation; anti-oxidants

Abbreviations

MS (Multiple sclerosis); GM (grey matter); WM (White matter); SOD (superoxide dismutase); ROS (reactive oxygen species); CNS (central nervous system); GPX (glutathione peroxidase); CAT (catalase); CSF (cerebral spinal fluid); MAL (Malondialdehyde); 4-HNE (4-hydroxynonenal); MBP (myelin basic protein); HLA (human leucocyte antigen).

1. Introduction

The cerebellum is a major site for tissue injury in multiple sclerosis (MS), particularly in patients with progressive disease (Calabrese et al., 2010; Kutzelnigg et al., 2007; Redondo et al., 2014). Indeed, cerebellar dysfunction in MS is a significant contributor to disability, commonly progressing regardless of treatment with disease-modifying agents (Waxman, 2005). Cerebellar dysfunction in MS is thought to arise due to a combination of white matter (WM) and grey matter (GM) injury, similar to that which occurs elsewhere in the cerebral cortex and underlying white matter tracts. The role of GM injury in MS has received less attention than WM injury, yet pathological studies of MS GM have revealed evidence for extensive demyelination, inflammation and neuronal loss (Kutzelnigg et al., 2005; Lucchinetti et al., 2011; Mahad et al., 2008). Furthermore, cortical atrophy on MRI scans is a well-recognised feature of established progressive disease (Steenwijk et al., 2016). Understanding the causes of MS GM injury in the cerebellum may help design therapies to reduce injury in this part of the brain.

A major role for reactive oxygen species (ROS) in the pathophysiology of MS and central nervous system (CNS) inflammatory disorders has been demonstrated (Cross et al., 1998; Smith et al., 1999; van Horssen et al., 2011). An imbalance in cellular redox homeostasis, leading to oxidative stress, may be caused by a large number of biological mechanisms resulting in the overproduction of ROS (Murphy, 2009). Changes leading to high concentrations of ROS have the potential to cause tissue damage and cell death within the CNS (Haider et al., 2011). Increases in ROS also trigger the formation of toxic molecules, such as lipid peroxidation products (Keller and Mattson, 1998), which themselves are strong reactive electrophiles capable of perpetuating oxidative stress (Abarikwu et al., 2012; Matveychuk et al., 2011). Experimentally, ROS and their reactive products cause cellular injury to neurons (and their axons) and oligodendrocytes (Abarikwu et al., 2012; French et al., 2009; Li et al., 2005; Wilkins and Compston, 2005). In both pathological studies and animal models of CNS inflammation, ROS play a key role in promoting tissue damage (Cross et al., 1998; Haider et al., 2011; Smith et al., 1999; van Horssen et al., 2008).

There is a complex cellular interplay between oxidative injury and anti-oxidant defences, and cells possess a diverse array of mechanisms to reduce ROS that build up during normal

physiological processes. These include anti-oxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and glutathione reductase. Failure of endogenous cell protection mechanisms that operate early in the disease course is postulated to be a major reason for on-going tissue damage in MS (van Horsen et al., 2011). Specifically, imbalances in the levels of oxidative stress molecules and anti-oxidant defences may be important in this respect, and precise identification of any imbalances may allow for the development of targeted therapeutic interventions which may help crucially tip the balance between cell death and survival. Indeed, a number of neurological conditions including amyotrophic lateral sclerosis, X-linked adrenoleucodystrophy and adrenomyeloneuropathy all highlight the important balance between oxidative stress and anti-oxidant defences (Moser et al., 2007; Rosen, 1993).

To date, studies have shown alterations in the levels of specific endogenous anti-oxidant enzymes and markers of oxidative stress in serum, cerebrospinal fluid (CSF) and brain tissue derived from patients with MS (Calabrese et al., 2002; Lund-Olesen, 2000; Tajouri et al., 2003; van Horsen et al., 2008). However, a clear understanding of precise mechanisms of tissue injury in MS is lacking, particularly in key pathological sites such as the cerebellum. In this study we have studied oxidative injury and the expression of anti-oxidant molecules in MS cerebellar grey matter.

2. Results

2.1 Antibody specificity

All primary antibodies used for immuno dot-blotting were tested for their specificity against their chosen antigens using western blotting techniques. Under the experimental conditions used, all antibodies displayed specific bands as described on manufacturer data sheets and/or relative to their reported molecular weights and were therefore considered suitable for use in immuno dot-blotting techniques (figures 2f, 3e, 5c).

2.2 Characterisation of cerebellar grey matter

Regions of demyelination (determined by myelin basic protein (MBP) staining) were seen in cerebellar grey matter (GM) tissue samples. Demyelination was typically seen exclusively within GM cerebellar above and below the layer of the Purkinje cells (supra- and infra-ganglionic layer) extending into the granular layer (cortical; figure 1a); or extending from white matter into the granular layer (leucocortical). As previously reported using the same patient cohort, all MS cases (and no control cases) showed areas of cortical and leucocortical demyelination within the grey matter. 29.4% (+/- 11.5 SEM) of the cerebellar cortex was demyelinated, representing 13.7% (+/- 8.5 SEM) with leucocortical demyelination and 15.7% (+/-7.2 SEM) with purely cortical demyelination (Redondo et al., 2014). Control sections immunolabelled for the macrophage/microglial marker human leucocyte antigen (HLA)-DP, DQ, DR, showed few positive cells in the cerebellum (figure 1b). In MS, HLA-DP, DQ, DR positive cells were scattered throughout cerebellar grey matter (GM) regions, where they had enlarged perikarya with thicker processes (figure 1c).

2.3 Quantification of tissue injury in cerebellar grey matter

Quantification of protein levels of HLA-DP, DQ, DR (determined by immunodot-blotting) revealed an increase (by approximately 2.5-fold) in MS cases compared to controls (Figure 2a). In addition immunodot-blotting of cerebellar GM tissue revealed a significant decrease in levels of MBP expression in keeping with immunohistochemical analysis of tissue (figure 2b).
Quantification of neuronal markers: microtubule-associated protein 2 (MAP2), neurofilament

200 (NF200) and calbindin-D28k levels by immunodot-blot analysis revealed a global reduction in all the markers tested, in MS cerebellar GM when compared to control tissue (Figure 2c-e).

2.4 Measurements of lipid peroxidation and oxidative stress

Malondialdehyde (MAL) and 4-hydroxynonenal (4-HNE) are major end-products of oxidation of polyunsaturated fatty acids, and are generally accepted as indicators of lipid peroxidation and oxidative stress (Requena et al., 1997). MAL and 4-HNE expression in the cerebellum of both control and MS patients was also investigated using immuno dot-blot analysis and immunohistochemistry. 4-HNE levels were significantly increased in the MS cerebellum grey matter when compared to control tissue (figure 3a). However, there were no significant changes evident in MAL levels between groups (Figure 3b).

Analysis of the relationship between 4-HNE with the corresponding anti-oxidant enzyme, neuronal or myelin expression in MS GM, revealed a significant negative correlation between the expression of 4-hydroxynonenal and MBP ($p < 0.05$; figure 3c). We found no correlation between the levels of MAL and MBP (figure 3d).

Areas of cerebellar tissue were subsequently immunolabelled using antibodies against 4-HNE and MAL in the sections derived from MS and control patients. In control sections, little or no positivity was demonstrable in the cerebellum (figure 4e-h). In MS cases, 4-HNE was abundant in the grey matter, particularly in the granular layer where it was co-localised with MBP labelling (figure 4a-d). In comparison, MAL showed less reactivity within the grey matter although did show occasional expression within neurons, such as Purkinje cells (data not shown).

2.5 Anti-oxidant enzyme gene and protein expression in cerebellar grey matter derived from MS and control patients

In order to determine anti-oxidant responses to the increased lipid peroxidation product 4-HNE, an analysis of anti-oxidant enzyme expression at the genomic and proteomic level using real-time polymerase chain reaction (RT-PCR) and immuno dot-blotting techniques respectively was performed in cerebellar GM in MS cases and controls. Both SOD1 and SOD2 mRNA and protein levels differed significantly between control and MS brain tissue, with a minimal 1.5 fold

increase in SOD expression in the MS brain (figures 5a,b). However, there were no significant changes evident in either catalase or GPX mRNA or protein levels between patient groups.

To investigate the cellular localisation of both SOD1 and SOD2 within the cerebellum grey matter, MS tissue was double immunolabelled using antibodies to either SOD1 or SOD2 in conjunction with HLA-DP, DQ, DR (figure 5d-k). In MS sections, neuronal localisation of both SOD1 and SOD2 expression was evident (particularly in the Purkinje cells which were identified on the basis of their characteristic morphology), showing typical neuronal cytoplasmic and mitochondrial labelling respectively, which in turn was relatively absent in the cells labelled with the macrophage/microglial marker HLA-DP, DQ, DR.

3. Discussion

We have performed a series of experiments using human cerebellar GM tissue investigating tissue injury, lipid peroxidation and anti-oxidant enzyme expression. In MS the GM was characterised by microglial infiltration, cortical and leucocortical demyelination, and a reduction in expression of neuronal markers. In addition, we show elevation of the lipid peroxidation product 4-HNE, which co-localises and negatively correlates with MBP expression in the granular layer of the cerebellum, suggesting 4-HNE may be relevant to GM demyelination in the cerebellum. Using quantitative PCR and immunodot-blotting assays, we have shown that anti-oxidant enzyme expression in the cerebellum is altered in MS at both the mRNA and protein level. We show here that superoxide dismuting enzymes (SOD1 and SOD2), localised within cerebellar neurons, are up-regulated in MS, but the activation of subsequent enzymes responsible for the detoxification of hydrogen peroxide, both catalase and GPX, are relatively deficient.

Several studies have highlighted the extent of tissue injury in MS cerebellum. In a similar way to the cerebral cortex, the cerebellar cortex appears a major site for demyelination in MS with one study reporting 38.7% of the cerebellar cortex being affected in a cohort of PPMS and SPMS patients (Kutzelnigg et al., 2007). It is thought that mechanisms of demyelination in the GM are similar to WM, although there may be particular topographical influences on GM demyelination. For instance, microglial infiltration into GM is commonly seen, but recent observations have suggested an association between meningeal inflammation and pathology in the adjacent cerebral cortex (Calabrese et al., 2015; Howell et al., 2014). The precise mechanism of GM demyelination needs further elucidation. In addition to myelin loss, other studies have previously reported neuronal changes within MS cerebellum. Purkinje cell densities in lesional cerebellar GM (compared to control) are reduced, but no significant reductions in Purkinje cell densities were seen in non-lesional GM (Kutzelnigg et al., 2007; Redondo et al., 2014). Changes in Purkinje cell morphology and neurofilament phosphorylation states are also to demyelination (Redondo et al., 2014). Our demonstration of global reductions in the expression of neuronal markers is in keeping with previous demonstration of neurodegenerative processes in MS.

Oxygen radical-induced cytotoxicity is associated with lipid peroxidation. By reacting with polyunsaturated fatty acids in the various cellular membranes, oxyradicals such as hydroxyl

(OH \cdot) and peroxynitrite (ONOO) give rise to a variety of lipid peroxidation products, including 4-hydroxynonenal and malondialdehyde, which are frequently measured as indicators of oxidative stress *in vivo* (Keller and Mattson, 1998; Requena et al., 1997). These reactive aldehydes are themselves cytotoxic; being strong reactive electrophiles and, amongst a plethora of toxic properties, they have the capability to inhibit DNA, RNA, and protein synthesis and may disrupt protein and membrane structures (reviewed by Matveychuk et al (Matveychuk et al., 2011)). They also have the capability to perpetuate oxidative stress by elevating mitochondrial ROS and inhibiting the antioxidant defence system through decreasing catalase, glutathione and SOD levels (Abarikwu et al., 2012; Long et al., 2009).

A major role for ROS in the pathophysiology of MS and central nervous system inflammatory disorders has been demonstrated in both pathological and experimental studies (Cross et al., 1998; Smith et al., 1999). Specifically, ROS may not only induce tissue injury and cellular death, but may also interfere with normal cellular functioning in the central nervous system. For instance, oxidative molecules, notably hydrogen peroxide, may disrupt oligodendrocyte maturation which may have consequences for endogenous repair mechanisms in MS (French et al., 2009). The enzymes responsible for the generation of ROS have been detected in tissue derived from patients with MS and experimental models of CNS inflammation. NADPH oxidase is responsible for the generation of superoxide ions and is highly expressed in activated microglia and infiltrating macrophages within MS lesions (Fischer et al., 2012). In addition, expression of iNOS, which is responsible for the generation of nitric oxide, is up-regulated in microglia and macrophages in acute and chronic lesions and has been linked to acute axonal injury (Bagasra et al., 1995; Diaz-Sanchez et al., 2006; Hill et al., 2004; Liu et al., 2001).

Profound oxidative damage to myelin, oligodendrocytes and neurons has also been demonstrated in MS lesions (Haider et al., 2011). ROS activation may also be a key event in the disruption of the blood-brain barrier, an important event in MS disease initiation (Schreibelt et al., 2006; Van der Goes et al., 2001). It is known that a number of oxidation products, including 4-HNE and oxidised phospholipids, are abundantly present in active MS lesions (Haider et al., 2011; van Horssen et al., 2008). Haider et al. demonstrated the presence of oxidative damage in MS lesions describing oxidized DNA (8-hydroxy-D-guanosine) and lipid peroxidation-derived structures

(malondialdehyde and oxidised phospholipid epitopes) (Haider et al., 2011). We have shown that 4-HNE levels are elevated in MS cerebellar GM. Within the MS cerebellum higher levels of 4-HNE expression are associated with reduced MBP expression, and that 4-HNE appears to be co-localised with myelin predominantly in the granular cell layer, suggesting that 4-HNE production within the GM may be associated with myelin loss.

The interactions between ROS and anti-oxidant enzymes within MS tissue are complex. Previous studies have shown changes in levels of certain endogenous anti-oxidant enzymes in MS tissue. For instance, increases in SOD1 gene transcription and protein expression have been detected in acute demyelinating MS lesions (Tajouri et al., 2003; van Horssen et al., 2008). These studies also showed evidence for changes in catalase expression by macrophages and some increases in GPX gene transcription (Tajouri et al., 2003; van Horssen et al., 2008). Whole genome arrays have also suggested that GPX isoenzyme transcripts may be differentially expressed in MS lesions (Fischer et al., 2012). Studies concerned with the determination of anti-oxidant enzymes in CSF of MS patients have also been limited. One small study has suggested SOD levels may be low in CSF derived from patients with MS (Lund-Olesen, 2000). Catalase activity has been found to be elevated (Calabrese et al., 2002) and conversely GPX has also been shown to be markedly decreased (Calabrese et al., 1994) in CSF samples from patients with MS compared to control.

This current study has shown increases in mRNA and protein expression of superoxide dismuting enzymes (SOD1 and 2) with no increase in hydrogen peroxide reducing enzymes (catalase and GPX). SOD1 and SOD2 are immunocytochemically localised to cerebellar neurons, and not infiltrating macrophage/microglial cells. Imbalances in the cellular redox homeostasis (increased ROS production alongside a lack in hydrogen peroxide detoxifying enzyme activity) may lead to an excess of hydrogen peroxide and the subsequent formation of 4-HNE and/or malondialdehyde as evident in this study. In support of this theory, *in vitro* studies have directly correlated decreased glutathione levels and GPX activity with increased lipid peroxidation and protein carbonylation (Bizzozero et al., 2006; Bizzozero et al., 2007). Our findings may therefore suggest that in MS the lack in activity of hydrogen peroxide detoxifying

enzymes during the inflammatory insult may conceivably play a pathogenic role in the accumulation of lipid peroxidation, oxidative stress and myelin injury in MS GM.

Taken together our observations provide evidence for tissue injury, lipid peroxidation and alterations in the production of protective anti-oxidant enzymes in MS GM. Generation of the lipid peroxidation product 4-HNE may be linked to myelin injury. Furthermore, imbalances in anti-oxidant enzyme expression in MS GM may be linked to abnormalities of ROS detoxification with subsequent build-up of toxic molecules causing cellular stress and irreversible tissue damage in patients with MS. Our data provide insights into oxidative and anti-oxidative processes occurring within the cerebellum in MS. This study suggests that directed and specific therapeutic strategies to reduce oxidative damage may play a crucial role in reducing tissue injury in MS.

4. Experimental procedure

4.1 Patients

Post-mortem cerebellum samples from eight patients with MS and six control patients were obtained through collaboration with the UK Multiple Sclerosis Tissue Bank at the Imperial College, London, UK. For the majority of MS cases, there were multiple tissue blocks for each patient, a total of twenty five frozen and six formalin-fixed paraffin embedded tissue blocks were therefore used in this study. All patients had been clinically diagnosed as having MS and this diagnosis had been confirmed during neuropathological autopsy examination. Control cerebellum samples were derived from patients who had died from causes other than neurological disease (Table 1). All tissues were collected with the donors' fully informed consent via a prospective donor scheme. At death, brains were removed; and either 'snap frozen' or fixed in neutral buffered formalin and tissue blocks embedded in paraffin. Case histories for all eight MS patients were reviewed (although some were incomplete). Six out of eight were documented to have moderate to severe ataxia.

4.2 Immunohistochemistry on paraffin sections

Cerebellar sections 10 µm in thickness were immunostained with antibodies to MBP (1:3200)(Serotec, Oxford, UK) and HLA-DP DQ DR (1:800)(Dako, Cambridgeshire, UK). Sections were deparaffinised in Clearene, dehydrated in 100% ethanol, hydrated in distilled water, and immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity, rinsed and microwaved in sodium citrate buffer (0.01 M, pH 6.0, 5 minutes) or EDTA buffer (1 mM, pH 8, 10 minutes) as appropriate and rinsed in phosphate-buffered saline (PBS). Non-specific binding was blocked with Vectastain blocking serum (20 minutes). After addition of the primary antibody, sections were incubated overnight at 4°C. The sections were then rinsed in PBS before incubation for 20 minutes with secondary antibody (Vectastain Biotinylated Universal antibody) and 20 minutes with VectaElite ABC Complex (PK-6200, Vector Laboratories, Peterborough, UK) followed by a 10-minute incubation with 3,3'-diaminobenzidine (DAB) and 0.01% H₂O₂. Sections were washed in water, immersed in copper sulphate DAB enhancer (4 minutes), counterstained with hematoxylin, dehydrated, cleared and mounted. Cerebellar slices (for 6 of the MS cases) were stained with MBP and measurements of

the regions of demyelination in each section obtained using Image J software to obtain the percentage of demyelination per standardized unit length. Further analysis of the tissue was performed using immuno-blotting techniques described below.

4.3 Immunofluorescence labelling on paraffin sections

Sections (10µm) were deparaffinised, hydrated and washed as above. To reduce auto-fluorescence, sections were incubated in 5 mM copper sulphate and 50 mM ammonium acetate for 1 h at room temperature prior to microwaving in sodium citrate buffer (0.01 M, pH 6.0, 5 minutes). Cells were labelled by double immunofluorescence using antibodies to 4-Hydroxynonenal (1:500)(Abcam, UK), Malondialdehyde (1:250)(Abcam, UK), MBP (1:100)(Serotec, Oxford, UK), HLA-DP DQ DR (1:250)(Dako, Cambridgeshire, UK), SOD1 (1:250)(Abcam, UK) and SOD2(1:250)(Abcam, UK). Non-specific binding was blocked with 10% normal goat serum diluted in PBS containing 0.1% triton. Sections were incubated at 4°C overnight with primary antibodies. Sections were then washed in PBS and incubated for 30 minutes in the dark with Alexa Fluor 488, goat anti-mouse or Alexa Fluor 555 (1:500), goat anti-rabbit (1:500)(Invitrogen, Paisley, UK), before being washed in PBS and mounted in Vectashield medium containing the nuclear dye 4'6'-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories).

4.4 Real-time Polymerase Chain Reaction (PCR)

Frozen tissue blocks were thawed on ice and grey matter carefully dissected away from white matter layers. RNA isolation, cDNA synthesis and RT-PCR were carried out as previously described by methods used within our laboratory (Gray et al., 2014; Hares et al., 2013). RT-PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Paisley, UK) with Assay-on-demand Gene Expression Products for SOD1, SOD2, catalase, GPX and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Taqman MGB probe, FAM dye-labelled, Applied Biosystems, Paisley, UK). The relative gene expression (RQ value) of a specific gene was calculated using the $2^{-\Delta\Delta C_t}$ method, and the mean taken for each group. Values were expressed relative to the housekeeping gene GAPDH in order to give an indication of the relative expression of genes throughout the whole tissue.

4.5 Immuno blotting

3.5.1 Sample preparation

Frozen tissue blocks were thawed on ice and grey matter carefully dissected away from white matter layers. The grey matter was then homogenized on ice by use of the PARIS kit (Ambion, UK) and a protease and phosphatase inhibitor cocktail (Thermo Scientific, UK) added at a dilution 1:100. The supernatants were removed and stored at -80°C until required.

4.5.2 Antibodies

Antibodies used were mouse anti-GAPDH (1:5000), rabbit anti-SOD1 (1:4000), mouse anti-SOD2 (1:4000), rabbit anti-catalase (1:10000), rabbit anti-GPX-1 (1:5000), rabbit anti-malondialdehyde (1:6000) and mouse anti-4 hydroxynonenal (1:300)(all from Abcam, UK); mouse anti-HLA-DP DQ DR (1:1000) (Dako, Cambridgeshire, UK); rat anti- MBP (1:2000) (Serotec, Oxford, UK); rabbit anti-calbindin D28k (1:2000), mouse anti-MAP2 (1:5000) and mouse anti-NF200 (1:10000)(all from Sigma-Aldrich, UK);

4.5.3 Western blotting and Immuno dot-blotting

Both western blotting and immune dot-blotting were carried out as previously described (Gray et al., 2014; Hares et al., 2013). Densitometric analysis of protein dots was performed using ImageJ software (NIH, USA). Values were expressed relative to the loading control protein GAPDH in order to give an indication of the relative expression of proteins throughout the whole tissue.

4.6 Statistical analysis

Statistical analysis was performed using a regression model using STATA v12. Where the distribution of the original variable differed significantly from normal, the regression model was fitted on the square root or log transformation of the response. The model also allowed for any correlation among multiple sections coming from the same brain tissue (cluster option). Non-parametric bootstrap was used to estimate standard errors and confidence intervals to account for possible non-normality of the parameter's distribution. Linear regression, Spearman's or Pearson's correlation methods were also used to analyse relationships between two variables. The analysis was performed using STATA v12 (Timberlake Consultants, London, UK) and

GraphPad Prism (GraphPad Software Inc, USA). For all tests, values of $p < 0.05$ were considered statistically significant.

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Conflict of Interest.

The authors declare that there is no conflict of interest.

Tables

Table 1 The characteristics of both MS and control patients used for this study

Figure legends

Figure 1 Demyelination and microglial infiltration in MS and control cerebellar grey matter. (a) MS section showing DAB (brown) labelling of myelin basic protein (MBP) demonstrating demyelination within the granular layer of MS cerebellum. The hatched areas in (i) represent the higher magnified images (ii/iii); (b) Control and (c) MS sections DAB (brown) immunolabelled with HLA-DP, DQ, DR showing an influx of microglial cells spread throughout the cerebellar grey matter in MS brain. (Scale bar = 100µm).

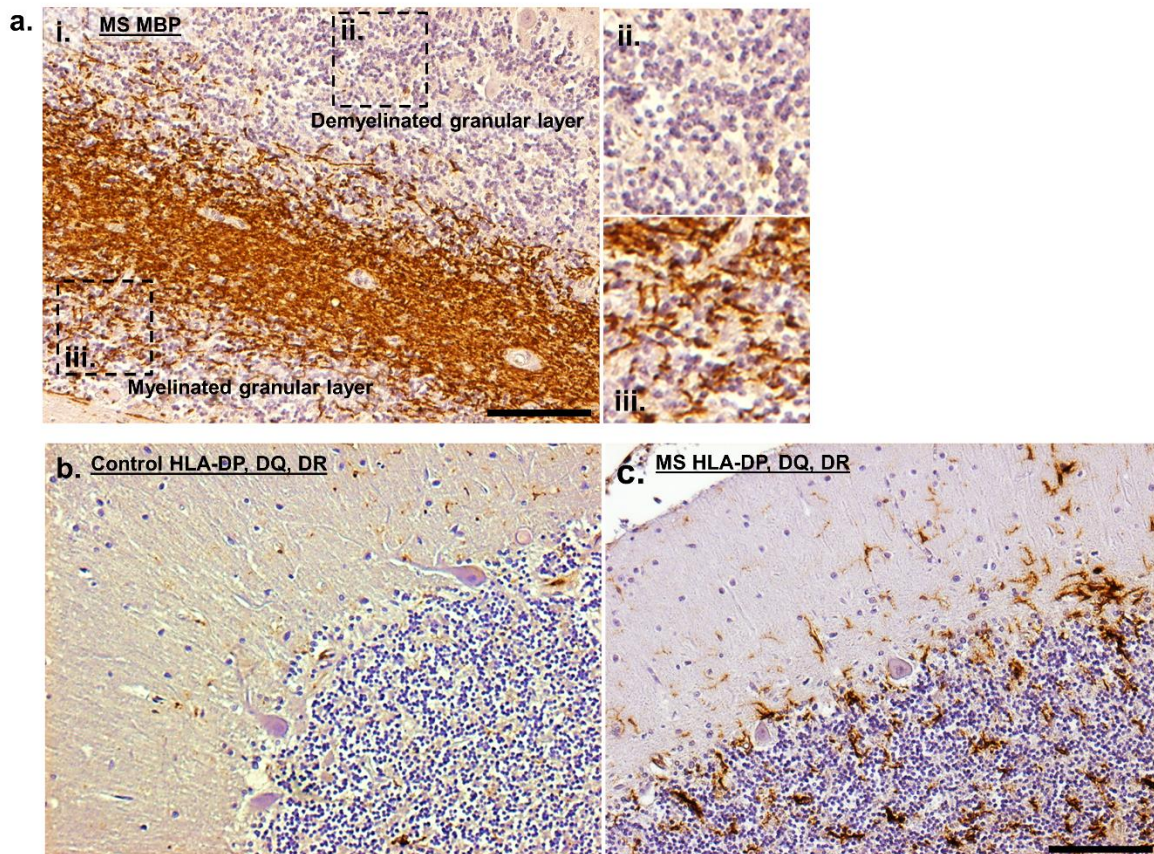


Figure 2 Quantification of cellular injury in control and MS cerebellar grey matter. The relative (a) HLA-DP, DQ, DR, (b) MBP, (c) Calbindin, (d) MAP2, and (e) NF200 protein expression in control and MS cerebellar GM samples determined by immuno dot-blot analysis when normalised to the protein expression of GAPDH (**p<0.01, ***p<0.001, comparing MS to control). (f) Western blot images depicting the specificity of the primary antibodies used for immunodot-blotting techniques against their chosen antigens.

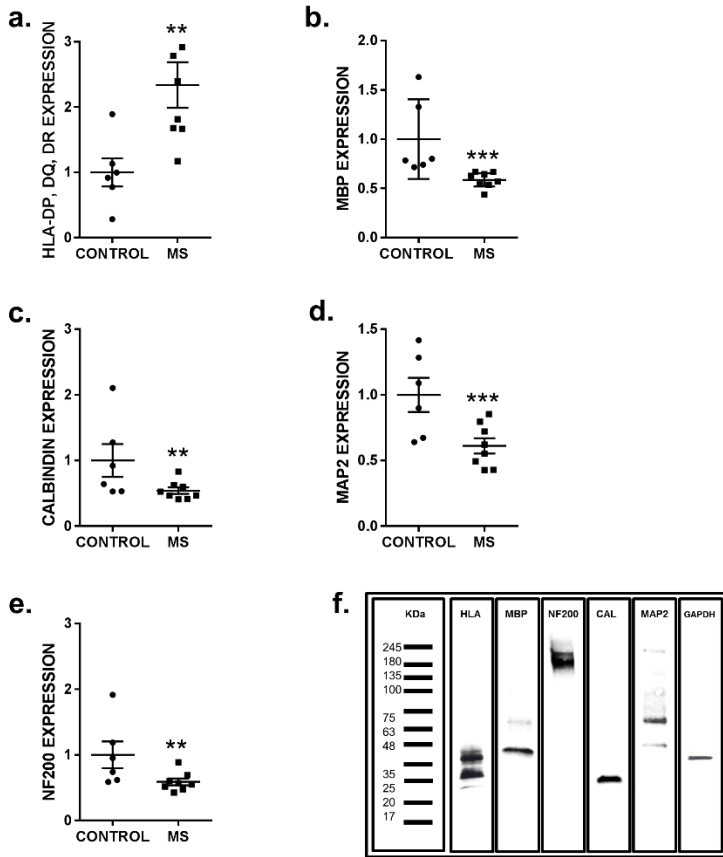


Figure 3 Indicators of lipid peroxidation and oxidative stress are increased in MS cerebellar grey matter. The relative (a) 4-hydroxynonenal and (b) malondialdehyde expression in control and MS cerebellar grey matter samples when normalised to the protein expression of GAPDH. Results are expressed as the mean +/- (SEM). (*p<0.05, ns (not significant), comparing MS to control). The correlation and linear regression analysis of:- 4-HNE levels with MBP (c) and MAL levels with MBP (d). All markers are normalised to GAPDH (for correlative analysis protein levels are expressed relative to the mean of the controls)(*p<0.05, ns (not significant); r = correlation coefficient). (e) Western blot images depicting the specificity of the primary antibodies used for immunodot-blotting techniques against their chosen antigens.

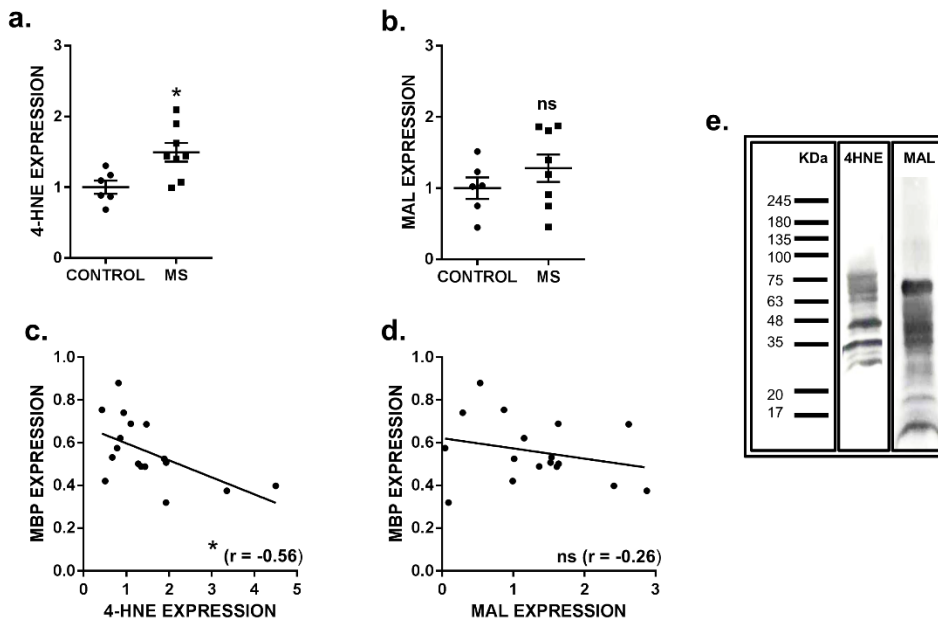


Figure 4 4-hydroxynonenal expression within MS cerebellar grey matter. Representative human cerebellar sections derived from an MS (a-d) and control (e-h) patient showing 4-hydroxynonenal is abundant throughout within the MS cerebellum (c) and co-localises with MBP (b). Sections are fluorescently labelled with DAPI nuclear stain (blue; a,e), MBP (red; b,f), 4-hydroxynonenal (green; c,g); (d,h) Merged figures. (Scale bar = 50µm).

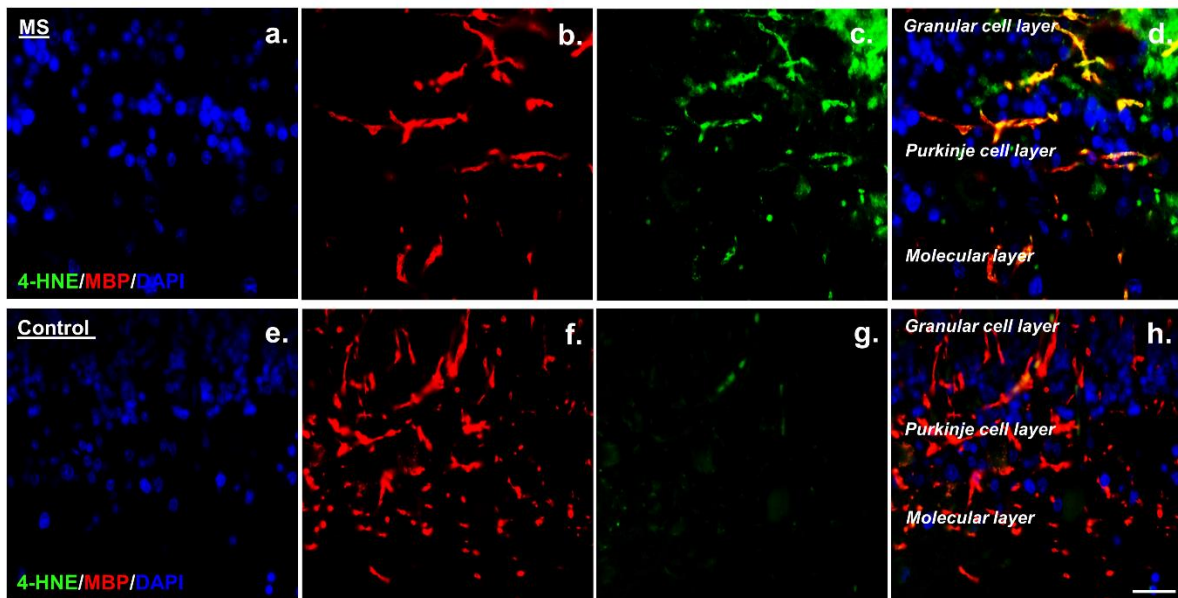
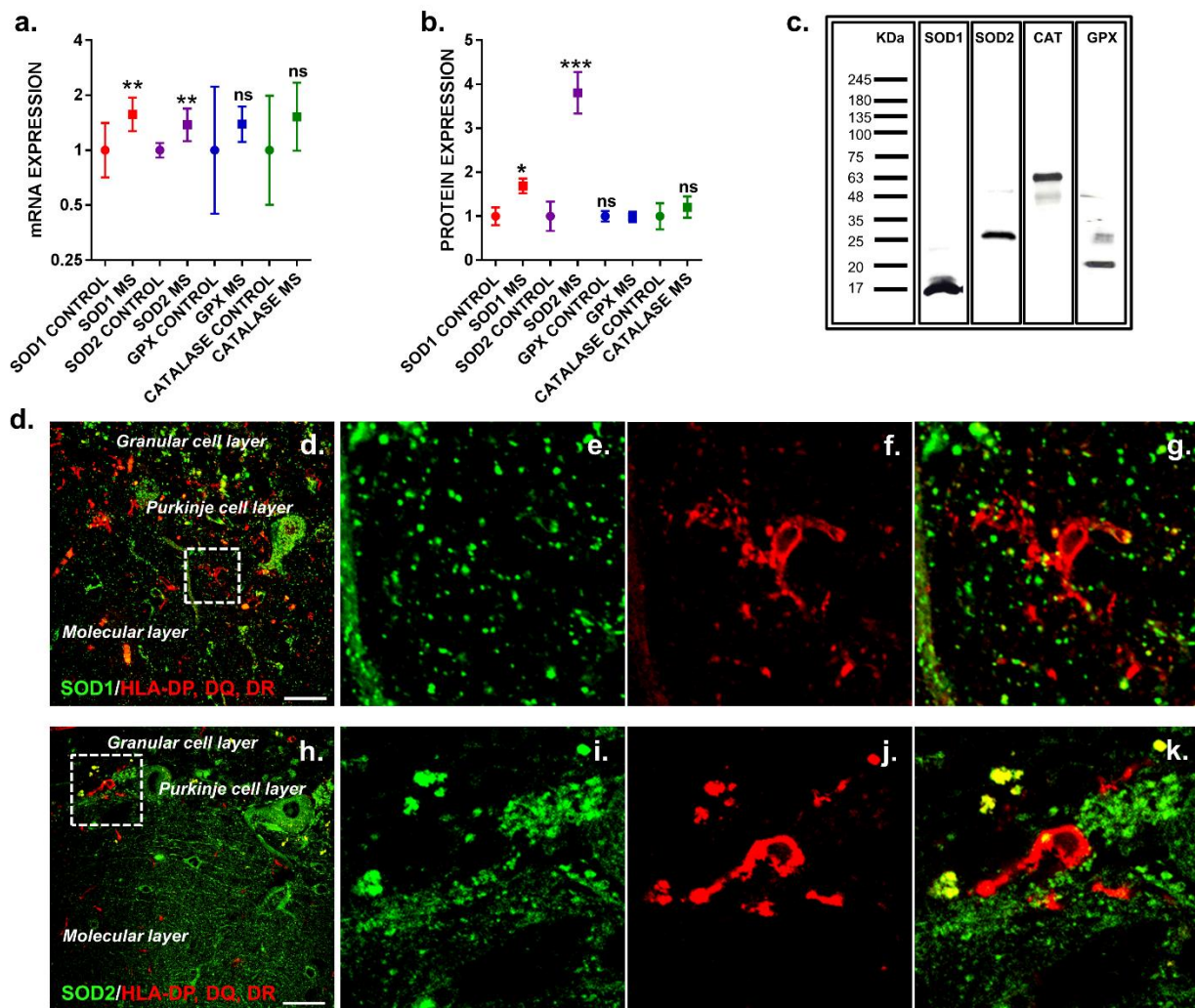


Figure 5 Anti-oxidant enzyme mRNA and protein expression levels in both control and MS cerebellar grey matter. The relative SOD1, SOD2, GPX and catalase (a) mRNA and (b) protein expression in control and MS cerebellar grey matter samples, determined by RT-PCR and immuno dot-blot respectively, when normalised to the house keeping gene GAPDH. Results are expressed as the mean +/- (SEM). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns (not significant), comparing MS to control). (c) Western blot images depicting the specificity of the primary antibodies used for immuno dot-blotting techniques against their chosen antigens. (d-k) Representative human cerebellar sections derived from MS patients showing neuronal localisation of both SOD1 (green; d-g) and SOD2 (green; h-k), which is relatively absent in HLA-DP, DQ, DR (red) positive cells. The hatched area in (d) represents the higher magnified images (e, f, g). The hatched area in (h) represents the higher magnified images (i, j, k). (Scale bar = 50 μ m).



References

- Abarikwu, S.O., Pant, A.B., Farombi, E.O., 2012. 4-Hydroxynonenal induces mitochondrial-mediated apoptosis and oxidative stress in SH-SY5Y human neuronal cells. *Basic Clin Pharmacol Toxicol.* 110, 441-8.
- Bagasra, O., Michaels, F.H., Zheng, Y.M., Bobroski, L.E., Spitsin, S.V., Fu, Z.F., Tawadros, R., Koprowski, H., 1995. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proc Natl Acad Sci U S A.* 92, 12041-5.
- Bizzozero, O.A., Ziegler, J.L., De Jesus, G., Bolognani, F., 2006. Acute depletion of reduced glutathione causes extensive carbonylation of rat brain proteins. *J Neurosci Res.* 83, 656-67.
- Bizzozero, O.A., Reyes, S., Ziegler, J., Smerjac, S., 2007. Lipid peroxidation scavengers prevent the carbonylation of cytoskeletal brain proteins induced by glutathione depletion. *Neurochem Res.* 32, 2114-22.
- Calabrese, M., Mattisi, I., Rinaldi, F., Favaretto, A., Atzori, M., Bernardi, V., Barachino, L., Romualdi, C., Rinaldi, L., Perini, P., Gallo, P., 2010. Magnetic resonance evidence of cerebellar cortical pathology in multiple sclerosis. *J Neurol Neurosurg Psychiatry.* 81, 401-4.
- Calabrese, M., Magliozzi, R., Ciccarelli, O., Geurts, J.J., Reynolds, R., Martin, R., 2015. Exploring the origins of grey matter damage in multiple sclerosis. *Nat Rev Neurosci.* 16, 147-58.
- Calabrese, V., Raffaele, R., Cosentino, E., Rizza, V., 1994. Changes in cerebrospinal fluid levels of malondialdehyde and glutathione reductase activity in multiple sclerosis. *Int J Clin Pharmacol Res.* 14, 119-23.
- Calabrese, V., Scapagnini, G., Ravagna, A., Bella, R., Foresti, R., Bates, T.E., Giuffrida Stella, A.M., Pennisi, G., 2002. Nitric oxide synthase is present in the cerebrospinal fluid of patients with active multiple sclerosis and is associated with increases in cerebrospinal fluid protein nitrotyrosine and S-nitrosothiols and with changes in glutathione levels. *J Neurosci Res.* 70, 580-7.
- Cross, A.H., Manning, P.T., Keeling, R.M., Schmidt, R.E., Misko, T.P., 1998. Peroxynitrite formation within the central nervous system in active multiple sclerosis. *J Neuroimmunol.* 88, 45-56.

- Diaz-Sanchez, M., Williams, K., DeLuca, G.C., Esiri, M.M., 2006. Protein co-expression with axonal injury in multiple sclerosis plaques. *Acta Neuropathol.* 111, 289-99.
- Fischer, M.T., Sharma, R., Lim, J.L., Haider, L., Frischer, J.M., Drexhage, J., Mahad, D., Bradl, M., van Horssen, J., Lassmann, H., 2012. NADPH oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury. *Brain.* 135, 886-99.
- French, H.M., Reid, M., Mamontov, P., Simmons, R.A., Grinspan, J.B., 2009. Oxidative stress disrupts oligodendrocyte maturation. *J Neurosci Res.* 87, 3076-87.
- Gray, E., Kemp, K., Hares, K., Redondo, J., Rice, C., Scolding, N., Wilkins, A., 2014. Increased microglial catalase activity in multiple sclerosis grey matter. *Brain Res.* 1559, 55-64.
- Haider, L., Fischer, M.T., Frischer, J.M., Bauer, J., Hoftberger, R., Botond, G., Esterbauer, H., Binder, C.J., Witztum, J.L., Lassmann, H., 2011. Oxidative damage in multiple sclerosis lesions. *Brain.* 134, 1914-24.
- Hares, K., Kemp, K., Rice, C., Gray, E., Scolding, N., Wilkins, A., 2013. Reduced axonal motor protein expression in non-lesional grey matter in multiple sclerosis. *Mult Scler.*
- Hill, K.E., Zollinger, L.V., Watt, H.E., Carlson, N.G., Rose, J.W., 2004. Inducible nitric oxide synthase in chronic active multiple sclerosis plaques: distribution, cellular expression and association with myelin damage. *J Neuroimmunol.* 151, 171-9.
- Howell, O.W., Schulz-Trieglaff, E.K., Carassiti, D., Gentleman, S.M., Nicholas, R., Roncaroli, F., Reynolds, R., 2014. Extensive grey matter pathology in the cerebellum in multiple sclerosis is linked to inflammation in the subarachnoid space. *Neuropathol Appl Neurobiol.*
- Keller, J.N., Mattson, M.P., 1998. Roles of lipid peroxidation in modulation of cellular signaling pathways, cell dysfunction, and death in the nervous system. *Rev Neurosci.* 9, 105-16.
- Kutzelnigg, A., Lucchinetti, C.F., Stadelmann, C., Bruck, W., Rauschka, H., Bergmann, M., Schmidbauer, M., Parisi, J.E., Lassmann, H., 2005. Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain.* 128, 2705-12.
- Kutzelnigg, A., Faber-Rod, J.C., Bauer, J., Lucchinetti, C.F., Sorensen, P.S., Laursen, H., Stadelmann, C., Bruck, W., Rauschka, H., Schmidbauer, M., Lassmann, H., 2007. Widespread demyelination in the cerebellar cortex in multiple sclerosis. *Brain Pathol.* 17, 38-44.

- Li, J., Baud, O., Vartanian, T., Volpe, J.J., Rosenberg, P.A., 2005. Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes. *Proc Natl Acad Sci U S A.* 102, 9936-41.
- Liu, J.S., Zhao, M.L., Brosnan, C.F., Lee, S.C., 2001. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am J Pathol.* 158, 2057-66.
- Long, J., Liu, C., Sun, L., Gao, H., Liu, J., 2009. Neuronal mitochondrial toxicity of malondialdehyde: inhibitory effects on respiratory function and enzyme activities in rat brain mitochondria. *Neurochem Res.* 34, 786-94.
- Lucchinetti, C.F., Popescu, B.F., Bunyan, R.F., Moll, N.M., Roemer, S.F., Lassmann, H., Bruck, W., Parisi, J.E., Scheithauer, B.W., Giannini, C., Weigand, S.D., Mandrekar, J., Ransohoff, R.M., 2011. Inflammatory cortical demyelination in early multiple sclerosis. *N Engl J Med.* 365, 2188-97.
- Lund-Olesen, K., 2000. Etiology of multiple sclerosis: role of superoxide dismutase. *Med Hypotheses.* 54, 321-2.
- Mahad, D., Ziabreva, I., Lassmann, H., Turnbull, D., 2008. Mitochondrial defects in acute multiple sclerosis lesions. *Brain.* 131, 1722-35.
- Matveychuk, D., Dursun, S.M., Wood, P.L., Baker, G.B., 2011. Reactive Aldehydes and Neurodegenerative Disorders. *Klinik Psikofarmakoloji Bulteni-Bulletin of Clinical Psychopharmacology.* 21, 277-288.
- Moser, H.W., Mahmood, A., Raymond, G.V., 2007. X-linked adrenoleukodystrophy. *Nat Clin Pract Neurol.* 3, 140-51.
- Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. *Biochem J.* 417, 1-13.
- Redondo, J., Kemp, K., Hares, K., Rice, C., Scolding, N., Wilkins, A., 2014. Purkinje Cell Pathology and Loss in Multiple Sclerosis Cerebellum. *Brain Pathol.*
- Requena, J.R., Fu, M.X., Ahmed, M.U., Jenkins, A.J., Lyons, T.J., Baynes, J.W., Thorpe, S.R., 1997. Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. *Biochem J.* 322 (Pt 1), 317-25.
- Rosen, D.R., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature.* 364, 362.

- Schreibelt, G., Musters, R.J., Reijkerkerk, A., de Groot, L.R., van der Pol, S.M., Hendrikx, E.M., Dopp, E.D., Dijkstra, C.D., Drukarch, B., de Vries, H.E., 2006. Lipoic acid affects cellular migration into the central nervous system and stabilizes blood-brain barrier integrity. *J Immunol.* 177, 2630-7.
- Smith, K.J., Kapoor, R., Felts, P.A., 1999. Demyelination: the role of reactive oxygen and nitrogen species. *Brain Pathol.* 9, 69-92.
- Steenwijk, M.D., Geurts, J.J., Daams, M., Tijms, B.M., Wink, A.M., Balk, L.J., Tewarie, P.K., Uitdehaag, B.M., Barkhof, F., Vrenken, H., Pouwels, P.J., 2016. Cortical atrophy patterns in multiple sclerosis are non-random and clinically relevant. *Brain.* 139, 115-26.
- Tajouri, L., Mellick, A.S., Ashton, K.J., Tannenber, A.E., Nagra, R.M., Tourtellotte, W.W., Griffiths, L.R., 2003. Quantitative and qualitative changes in gene expression patterns characterize the activity of plaques in multiple sclerosis. *Brain Res Mol Brain Res.* 119, 170-83.
- Van der Goes, A., Wouters, D., Van Der Pol, S.M., Huizinga, R., Ronken, E., Adamson, P., Greenwood, J., Dijkstra, C.D., De Vries, H.E., 2001. Reactive oxygen species enhance the migration of monocytes across the blood-brain barrier in vitro. *FASEB J.* 15, 1852-4.
- van Horssen, J., Schreibelt, G., Drexhage, J., Hazes, T., Dijkstra, C.D., van der Valk, P., de Vries, H.E., 2008. Severe oxidative damage in multiple sclerosis lesions coincides with enhanced antioxidant enzyme expression. *Free Radic Biol Med.* 45, 1729-37.
- van Horssen, J., Witte, M.E., Schreibelt, G., de Vries, H.E., 2011. Radical changes in multiple sclerosis pathogenesis. *Biochim Biophys Acta.* 1812, 141-50.
- Waxman, S.G., 2005. Cerebellar dysfunction in multiple sclerosis: evidence for an acquired channelopathy. *Prog Brain Res.* 148, 353-65.
- Wilkins, A., Compston, A., 2005. Trophic factors attenuate nitric oxide mediated neuronal and axonal injury in vitro: roles and interactions of mitogen-activated protein kinase signalling pathways. *J Neurochem.* 92, 1487-96.