Endoplasmic Reticulum Stress as a Component of Neurodegeneration in MS Grey Matter Lesions

Una Fitzgerald, Jill McMahon, Stephen McQuaid

2. Acknowledgements

Professor Richard Reynolds, UK Multiple Sclerosis Tissue Bank & Imperial College, London

Professor Michael Farrell, Beaumont Hospital, Dublin

Professor Hans Lassmann, Brain Research Institute, University of Vienna, Austria

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4. Introduction

The aim of this study was to investigate the role of endoplasmic reticulum (ER) stress in the development of grey matter (GM) lesions and neurodegeneration in Multiple Sclerosis (MS). In recent years, a growing number of investigators have reported a high frequency of GM lesions in MS, and these are associated with significant neuronal loss and subsequent cortical atrophy[1-5]. Unlike those occurring in white matter (WM), GM lesions are not associated with a classic inflammatory response and occur in the absence of large numbers of inflammatory cells and with very little deposition of complement or IgG [6, 7]. There is an increasing acceptance that the mechanisms underlying formation of WM lesions are essentially different from those resulting in GM lesion formation and this is reflected by the lack of correlation between white and grey matter pathology. GM demyelination and neuronal loss are substantial contributors to the disability and cognitive dysfunction observed in MS.

While there is extensive description of the potential molecular mechanisms underlying demyelination in white matter of brain, the pathogenesis of GM demyelination is poorly understood and in some cases its occurrence is under-reported, due to the difficulties in demonstrating grey matter demyelination. Some studies have suggested mitochondrial dysfunction to be a precursor to demyelination events, as cortical regions devoid of lesion indicators were found to have reduced levels of respiratory chain molecules [8]. Similarly, a role for nitric oxide in initiating axonal damage has been postulated since markers of NO signalling have been detected in GM lesions [9]. We propose that endoplasmic reticulum (ER) stress is another component of the "neurodestructive" environment that may play a crucial role in the neurone's decision to survive or die.

ER stress responses involve complicated intra-cellular signalling pathways (Fig 1) and these are usually triggered in response to a build up of unfolded or misfolded proteins within the ER. The Grp78/BiP molecule, normally bound to the trans-ER membrane molecular sensors PERK, IRE1 and ATF6, maintaining them in a quiescent state, will preferentially bind mis-folded proteins. This frees the molecular sensors allowing them to be activated, which in turn precipitates the events comprising the ER stress response. The reasons for build up of un- or mis-folded proteins within the ER in a cell are many, ranging from changes in the concentration of luminal calcium, affecting calcium-regulated proteins, to alterations in the function of redox-regulated proteins, caused by oxidative stress. Stress in the ER has been reported in a variety of chronic neurodegenerative disorders, including Alzheimer's Disease (AD), Parkinson's Disease and Amyotrophic Lateral Sclerosis [10-12]. Increased staining intensity of Grp78/BiP and PERK has been demonstrated in the brains of AD patients in comparison with normal controls[11]. Furthermore, several key molecular events reported to occur during ER perturbation, such as induction of death receptor DR5, up-regulation of the oxidase Ero1-a and down-regulation of the anti-apoptotic Bcl-2[13-15] may provide ways in which ER stress activation could bring about neuronal and oligodendrocyte cell death.

In a recently published immunohistochemical study on human MS tissue[16] we have demonstrated that the vast majority of active WM lesions displayed raised expression levels of the classical ER-associated transcription factor CHOP/GADD153. In particular, significant increases in expression were seen at the demyelinating edge of chronic active lesions when compared to control and chronic inactive lesions. Also, raised levels of ER-stress proteins were found to be present in oligodendrocytes,

astrocytes and T-cells from within perivascular infiltrates. These results have since been further substantiated at the RNA level[17] using laser capture microdissection and real-time PCR techniques. CHOP/GADD153 mRNA was shown to be significantly increased in the perilesional area of active WM plaques in comparison to normal appearing white matter (NAWM) from the same patients.

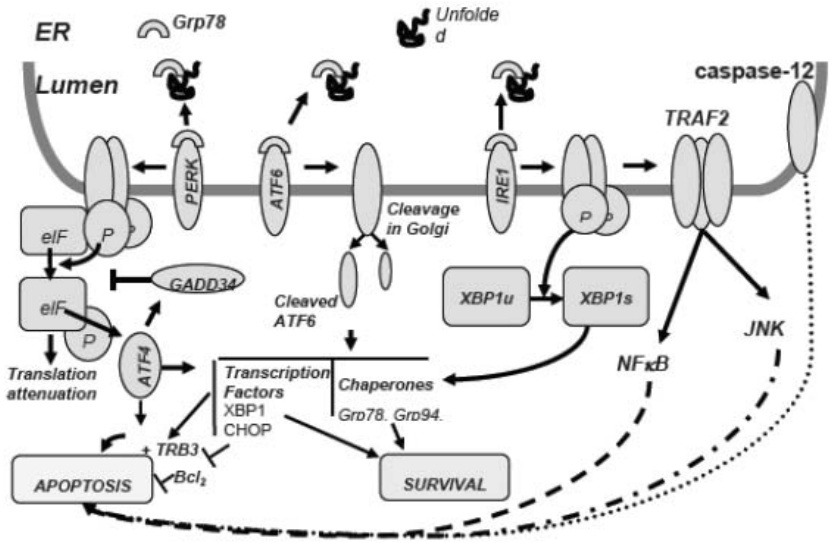

Figure 1.The unfolded protein response. When stress to the ER occurs, in the form of a build-up of unor mis-folded protein within the ER lumen, a response known as the unfolded protein response (UPR) is triggered. This is transduced via PERK, ATF6 & IRE1, 3 trans-membrane molecular sensors. The PERK arm leads to transient attenuation of protein translation & apoptosis via CHOP/GADD153 & TRB3. ATF6 mostly functions to up-regulate folding chaperones (Grp78 & Grp94 etc) and the ER stress-specific XBP1 transcription factor, as part of the protective response. Events downstream of Ire1 are complex and lead to activation of XBP1 followed by chaperone induction.

In terms of the development and progression of grey matter pathology in MS a number of cellular pathways are strongly associated with a stressed ER response:

1) neuronal or oligodendrocyte mitochondrial dysfunction with oxidative stress could cause a redox imbalance affecting redox-regulated foldases within the ER, causing a build up of misfolded proteins; 2) induced nitric oxide signalling may be amplified via the mitochondria and the ER in neurons and/or oligodendrocytes[18]; 3) B-cell differentiation has been shown to require the activation of the ER stress protein

XBP1[19] and so raised levels of XBP1 would be expected in the meningeal B-cell follicles recently described in MS[3] ; 4) activated microglia, the only immune cell reported to be present in type III MS lesions, are likely to contain high levels of ER stress proteins, as ultrastructural studies of macrophages in MS have shown the presence of trans-ER inclusions[20].

Investigation of the possible link between a perturbed ER and GM lesion development in MS will not only provide crucial information about the pathogenesis involved in the development of neurodegeneration but may also provide an important pathway which could be targeted in the development of new therapeutics.

5. **Methodology**

Patient material

Informed consent and local ethical approval was obtained for use of all brain tissue and for the conduct of this study. Case selection was retrospective and based on a confirmed neurological and neuropathological diagnosis of MS. It was also dependent on the availability of archival formalin-fixed paraffin-embedded (FFPE) MS brain blocks, showing grey matter pathology, from the UK Multiple Sclerosis Tissue Bank, Imperial College London (registered charity 207495). Tissue blocks, in which no GM pathology was seen, were also received from MS cases, as well as non-MS control blocks. A total of 12 GM-lesion blocks, 14 normal appearing grey matter (NAGM) blocks and 12 non-MS controls were used. This information, along with the anatomical areas sampled, age, sex, disease duration, death-autopsy interval and cause of death are summarised in Tables 1a and 1b below.

Immunohistochemical staining

Initially, it was planned to use the Ventana Discovery automated staining system for all immunohistochemical staining. However, initial optimisation work carried out in the Queen's University of Belfast lab demonstrated that manual immunostaining resulted in equally high levels of reproducibility and good quality staining as that produced by the automated system.

Table 1a. Demographic, clinical and sampling information of MS cases used.

Case No	GM Lesion	Block Area	NAGM	Block Area	Age (y)/Sex	Disease Duration	Death-autopsy interval(h)	Cause of Death
MS 80	√	PVC	√	PL	71/F	35	24	Heart failure
MS 85	√	PVC	√	PL	59/F	35	34	Cerebrovascular disease. MS
MS 99	√	PVC	√	PL	81/F	41	23	MS
MS 100	√	PVC	√	PL	46/M	8	7	Pneumonia
MS 104	√	PVC	√	SFG	53/M	11	12	MS. Urinary tract infection
MS 121	√	PVC	–	–	49/F	14	24	MS
MS 122	√	PVC	–	–	44/M	NK	16	Bronchopneumonia
MS 157	√	PVC	√	SFG	39/F	19	12	MS
MS 160	√	PVC	√	CG	44/F			a
MS 180	√	PVC	√	PCG				
MS 197	√	PVC	√	PL	51/F	NK	10	Bronchopneumonia
MS 255	√	PVC	–	–	45/M	25	24	Bronchopneumonia, MS
MS 326	–	–	√	SFG	62/M	32	24	MS, prostate cancer
MS 333	–	–	√	SFG	82/F	46	24	Septicaemia, MS
MS 333	–	–	√	TC	53/F	19	17	Sepsis, aspiration pneumonia
MS 340	–	–	√	TC	49/M			M'static pancre
MS 347	–	–	√	PVC				

GM =grey matter. NAGM=normal-appearing grey matter. PVC= primary visual cortex, PL=parietal lobe, SFG=superior frontal gyrus, CG=cingulate gyrus, PCG =precentral gyrus, TC= temporal cortex, –=not applicable

= awaiting clinical details from UK MS Tissue Bank

Table 1b: Demographic, clinical and sampling information of control cases used.

Case No	Age (y)/ sex	Clinical diagnosis	Death-autopsy interval (h)	Cause of death
C 29	71/M	NA	33	CML, abdominal haemorrhage, ruptured spleen
C30	75/M	NA	17	CVA, aspiration pneumonia
C32	88/M	NA	22	Prostate cancer, bone metastases
C36	68/M	NA	30	Cor pulmonale heart failure, fibrosing alveolitis, c.a. atheroma
C37	84/M	NA	5	Bladder cancer, pneumonia
C39	82/M	NA	21	Myelodysplastic syndrome/rheumatoid arthritis
C41	54/M	NA	20	Lung cancer
C43				
C45				
C46				
C48				
C49				

= awaiting clinical details from UK MS Tissue Bank

Slides from all blocks have subsequently been stained manually with the following:

1. Histochemical stain (LFB/H&E) to specifically stain myelin and demonstrate general tissue architecture.
2. Immunohistochemical staining
    - (i) myelin oligodendrocyte glycoprotein (MOG) antibody. This specifically stains myelin and is much more sensitive than the LFB/H&E stain. This is particularly useful for detection of demyelination in grey matter, which is often difficult to demonstrate using more conventional myelin stains.
    - (ii) HLA-DR. This antibody stains activated microglia and allows classification of WM MS lesions
    - (iii) CHOP/GADD153. ER-stress associated antibody
    - (iv) Bip/Grp78, ER-stress associated antibody
    - (v) D110. This antibody indicates hypoxia in brain tissue.
    - (vi) βAPP (β-amyloid precursor protein). This antibody demonstrates axonal injury.
    - (vii) RT97 and SMI32. These antibodies stain neurofilaments and may aid in the identification of neurodegeneration.

Colorimetric immunohistochemistry was carried out using the following technique:

All sections were de-paraffinized and endogenous peroxidase blocked by incubation in hydrogen peroxidase in 100% ethanol. If antigen retrieval was required (see Table 2) this was performed in 0.01M Tris-EDTA (pH 9.0) in a pressure cooker at full steam pressure for 3min.

D110 sections were pretreated with 10% fetal calf serum (Invitrogen, Ireland) in TBS for 20min at room temperature. All antibodies were incubated on sections at 4^{o}C overnight, detected using peroxidase-labelled Envision^{TM} anti-mouse or anti-rabbit secondary antibodies (Dako), with diaminobenzidine (Dako) as chromogen, and counterstained in haematoxylin. Sections were then dehydrated in graded alcohol, cleared in xylene and mounted in DPX (VWR).

Table 2: Primary Antibodies

	Antibody	Supplier	Isotype	Species	Dilution	Ag Retrieval
Myelin	MOG	Gift *	Monoclonal	M	1/100	√
Activated microglia	HLA-DR	Dako	Monoclonal	M	1/400	√
Neurofilaments	SMI 32	Covance	Monoclonal	M	1/1000	√
Neurofilaments	RT97	Millipore	Monoclonal	M	1/500	√
Axonal damage	APP	Dako	Monoclonal	M	1/2000	√
Hypoxia	D110	Gift†	Monoclonal	M	1/2000	√
ER-stress-associated molecule	BiP	Santa Cruz	Polyclonal	R	1/200	√
ER-stress-associated molecule	CHOP	Santa Cruz	Polyclonal	R	1/400	√

*From Prof R Reynolds, Imperial College London

† From Prof Hans Lassmann, Institute for Brain Research, University of Vienna, Austria

Histological characterisation

All slides were analysed initially using LFB/H&E to assess the overall pathological features such as the number and extent of demyelinated lesions, white matter demyelination, grey matter demyelination (where visible), inflammation and/or perivascular cuffing.

MOG-immunostained slides were then used to detect GM demyelination in greater detail and to classify the GM lesions present.

All grey matter lesions have been classified into Types 1-III using the following criteria [21]:

- Type I lesions: grey matter demyelination which extends into the sub-cortical white matter

- Type II lesions: lesions contained entirely within the cortex and not in contact either with the pia matter or the sub-cortical white matter.

- Type III lesions: sub-pial lesions extending from the pia matter into the cortex but not in contact with the sub-cortical white matter.

Examples of all three types are shown in Figure 2.

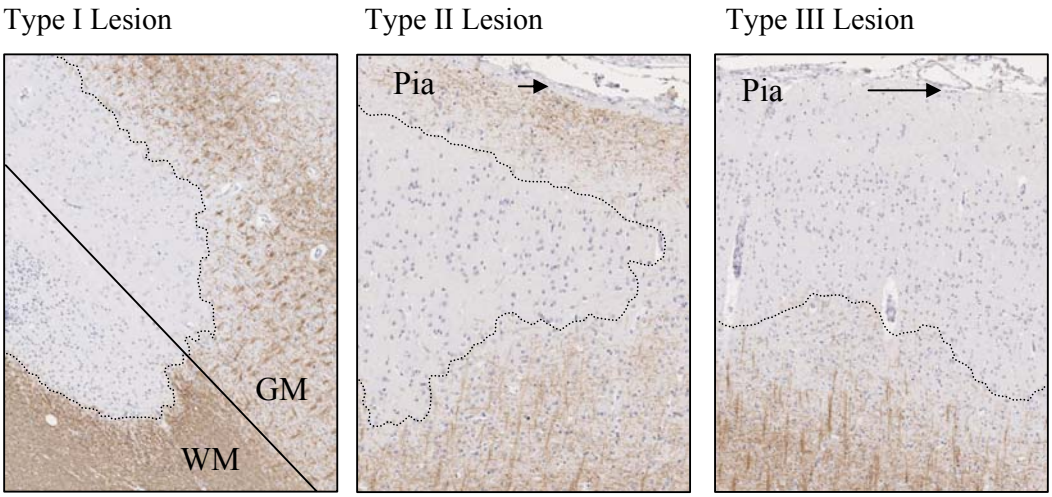

Figure 2: Classification of GM lesions. Type 1 lesions cross the grey matter (GM) – white matter (WM) boundary. Type II lesions are entirely within cortex. Type IIi lesions are sub-pial and don't contact WM.

Slides stained with HLA-DR, βAPP, RT97 and SMI32 antibodies have also been analysed within areas of grey matter demyelination.

Immunohistochemical scoring

An initial semi-quantitative grading of staining for hypoxia and ER-stress-associated molecules, within both grey and white matter, has been carried out on all blocks. Representative images of staining from within, and adjacent to, different types of MS grey matter lesions have been collected. Corresponding areas from NAGM and non-MS grey matter, from within the same cortical layers, have also been imaged.

In order to quantify further the number of cells in the cortex expressing CHOP and D110, in and around grey matter lesions, a more specific system was employed which attempted to compensate for the different cell densities and types which occur in cortical layers 1 to 6. Tissue sections were scanned at magnification x400, starting from the pia mater and across into the sub-cortical white matter. This comprised four fields, at this magnification whereby cortical layers 1, 2 and 3a were seen in field 1, cortical layer 3b and 4 in field 2, layers 5 and 6 in field 3 and field 4 showed adjacent sub-cortical white matter (see Figure 3). Areas of cortex comprising a greater or smaller number of microscope fields were not used for this type of quantification. The number of D110- or CHOP-positive cells was counted in each of these fields, and an average of 3 fields taken at each location. In the case of small, discrete demyelinated areas being present in any or all of the cortical layers, counts were also made in adjacent myelinated areas. If the white matter areas were also demyelinated, counts were carried out in normally-myelinated adjacent white matter areas.

Similar cell counts were performed on stained slides from lesion-free normal appearing grey matter from MS patients, and grey matter of non-MS controls.

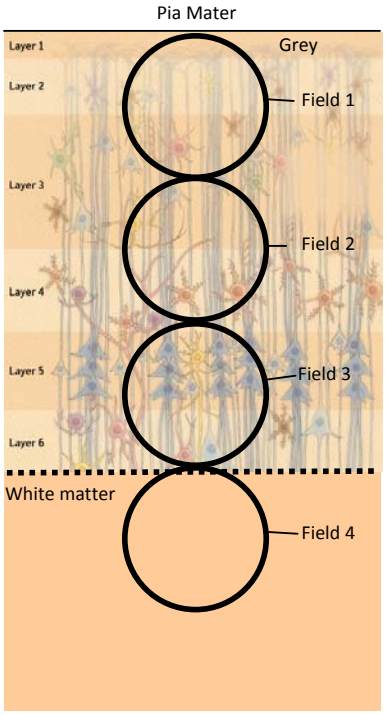

Figure 3: Immunohistochemical scoring across cortical layers. Using magnification of x400, 4 fields were examined. Fields 1 to 3 include cortical layers 1 to 6. Field 4 resides in the adjacent white matter.

Immunopositive cell counts were carried out either on images obtained using an Olympus BX51 microscope and Olympus DP70 colour camera or on sections which were digitally-scanned using an Aperio Scanscope T3 (www.aperio.com) with a 40x objective.

6. Literature Review

Multiple sclerosis (MS) is a disease of the central nervous system, characterised by multi-focal plaques (or areas of demyelination). Although grey matter demyelination in MS was described as early as 1916, it has been widely considered to be a disease of the white-matter. This opinion has arisen largely due to the inadequacies of commonly-used histological stains (such as Luxol Fast Blue[22, 23]) to stain adequately the myelin in normal grey matter rendering it very difficult to identify cortical demyelination on tissue sections. Similarly, grey matter lesions lack conspicuity when using conventional magnetic resonance imaging (MRI) techniques, making them easy to overlook. However, with the advent of immunocytochemical techniques to detect myelin proteins, and hence a much more sensitive means of staining myelin1, and advances in MRI technology, e.g. development of three-dimensional double inversion recovery (3D DIR)[24], magnetic transfer ratio (MTR)[25], diffusion tensor imaging (DTI)[26] and 3D T1-based imaging, the interest in lesions of the grey matter was renewed and it was realised that the extent of grey matter pathology in the disease had been greatly underestimated. This may account for the "clinico-radiological paradox"[27] whereby the clinical course of disease and extent of disability often shows little correlation to the number of white matter lesions detected by conventional MRI techniques.

It is now known that there is widespread cortical demyelination in MS, which is thought to comprise a mean of between 9 and 26% [1, 5, 28]of the grey matter volume, but relatively little is known about the nature and pattern of these plaques. Unlike demyelinated areas in the white matter, grey matter lesions are not visible macroscopically, and rarely show the hallmarks of white matter lesions such as deposition of complement, lymphocytic infiltration and disruption of the blood brain barrier (BBB). Instead, these lesions seem to be restricted to showing myelin loss and, in some cases, a minor microglial reaction or sometimes loss of cells (neurones and

glia) and transection of axons[1, 29]. As a result it is fairly widely accepted that the mechanisms underlying the formation of grey matter lesions may be intrinsically different from those that bring about white matter demyelination.

In general, the pathogenesis involved in grey matter demyelination is very poorly understood, as are the events leading to neuronal cell death and cortical thinning. Mitochondrial dysfunction[8] and increased levels of nitric oxide[9] have both been proposed as having either precursory or causal effects in GM lesion development and subsequent cell death and we hypothesise that endoplasmic reticulum (ER) stress may play an important part in the development of a demyelinating and neurodestructive environment.

ER stress responses involve complicated intra-cellular signalling pathways and these are usually triggered in response to a build up of unfolded or misfolded proteins within the ER due to various factors such as changes in luminal calcium concentration and alterations in redox-regulated protein function occurring as a response to oxidative stress. However, ER stress –associated molecules have also been implicated in the activation and differentiation of macrophages in response to danger signals[30] and it is possible that the "quiet" innate immune responses, mediated by brain-resident microglia in grey matter lesions, may require ER stress molecules to bring about protective and non-protective responses to a perturbed environment.

Stress in the ER has been reported in a variety of chronic neurodegenerative disorders, including Alzheimer's Disease (AD), Parkinson's Disease and Amyotrophic Lateral Sclerosis[10-12] and several key molecular events, reported to occur as a result ER perturbation, such as induction of death receptor DR5, up-regulation of the oxidase Ero1-a and down-regulation of the anti-apoptotic molecule Bcl-2[13-15] may provide a means by which ER-stress activation could bring about neuronal and glial cell death.

Grey matter lesions have variously been classified into 7 distinct types, assuming a causal relationship to the anatomy of the vasculature[31] and 3 types, based on location within the cortex as determined using immunocytochemical detection of myelin,[1, 6] but the latter classification is most commonly used. We have analysed an array of grey matter lesions in twelve cases of MS and carried out a comprehensive immunocytochemical study to detect the presence of ER stress-associated molecules BiP and CHOP, as well as the hypoxia-associated D110 molecule. This data was correlated to grey matter lesion type (using MOG staining and LFB) and any relationship between pathology and upregulation of protein

expression noted. Blocks of normal-appearing grey matter from fifteen MS cases, and from twelve non-MS controls, were also analysed.

7. **Findings and Discussion**

Identification of Grey Matter Pathology

The initial part of this study involved analysis of all the tissue sections received, using LFB/H&E histochemical stain and myelin oligodendrocyte glycoprotein (MOG) antibody. From the outset it was evident that, while white matter (WM) lesions were easily identified using the histochemical stain, many of the areas of cortical demyelination areas were almost impossible to see, especially at the lower magnifications used to scan the slides. This is primarily because of the poor staining of grey matter (GM) myelin by LFB, even in areas subsequently recognised as being normal-appearing grey matter (NAGM). Hence, myelin loss, especially in sub-pial areas, is particularly difficult to recognise.

This problem was completely resolved with the use of MOG immunohistochemistry which resulted in very clear labelling of myelin, both in normal-appearing areas and areas of cortical pathology. Using this methodology it was easy to establish "normal" myelination levels in different cortical areas, making the phenomena of myelin thinning and myelin loss very easy to identify. This increase in sensitivity of immunochemical techniques over the more traditional LFB staining has previously been reported[22, 23].

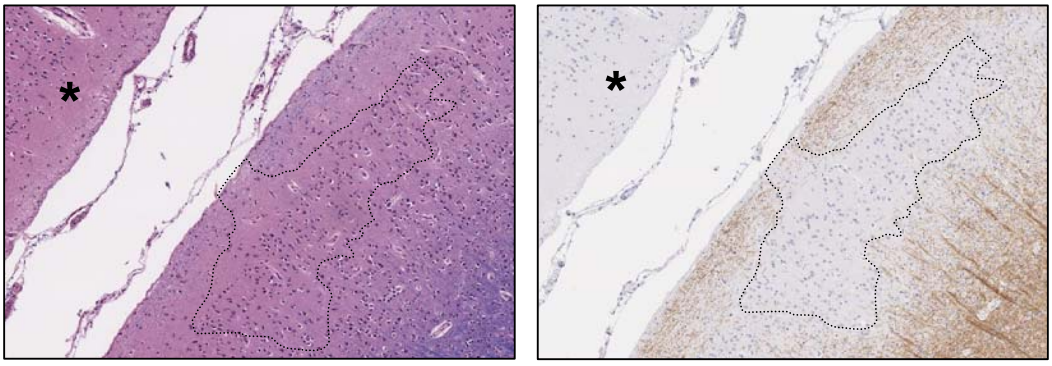

Figure 4. Comparison of LFB/H&E with MOG immunohistochemistry on serial sections. Absence of MOG staining (RH panel) clearly demarkates lesion which is not so clearly identified using LFB staining (LH panel).

All GM lesion were characterised into three types, using the criteria employed by Bo et al,

One interesting point observed while categorising the GM lesions was the lack of obvious HLA-DR +ve cells at the edge of the majority of lesions analysed. We have previously been able to identify active WM lesions at low magnifications because of the preponderance of activated microglia at lesion edges, which staining, in effect "outlines" the area of pathology, making it very easy to find. No such correlation was seen between HLA-DR positive activated microglia and the edges of GM demyelinated lesions, unless they were associated with WM lesions (Type I)

All three types of lesions were seen in this study, the majority of which were type III (sub-pial demyelination, not extending into the white matter). These lesions often were substantial in size, continuing along several gyri in length, as has previously been reported by other groups. On occasion, two of these types of lesion could be observed on either side of a particular gyrus, discontinuous from each other, but geographically separated only by the CSF between the two pial layers. This observation would seem to substantiate the theory that GM demyelination can arise as the result of chronic exposure of the surface of the brain to a cocktail of cytokines. These lesions seem mostly to be fairly quiescent, with little evidence of inflammation, or association with raised numbers of HLA-DR positive microglia

Type II lesions (those contained entirely within the GM), were seldom seen, and usually were small in size. These also seemed to be fairly quiescent in nature. Type I lesions (extending into the white matter) were often seen, though were not as common as Type III, and were very variable in size. These lesions were often seen to have HLA-DR positive cells at the edge, or nearby the lesions.

ER-stress and Hypoxia-Associated Molecules

Initial assessment of CHOP and BiP staining was carried out on all groups of slides (GM pathology, NAGM and non MS control) using a semi-quantitative scheme, whereby slides were graded on a scale of + to ++++ depending on the number and intensity of staining in positive cells, both in GM and WM areas. These results are shown below (Figure 5).

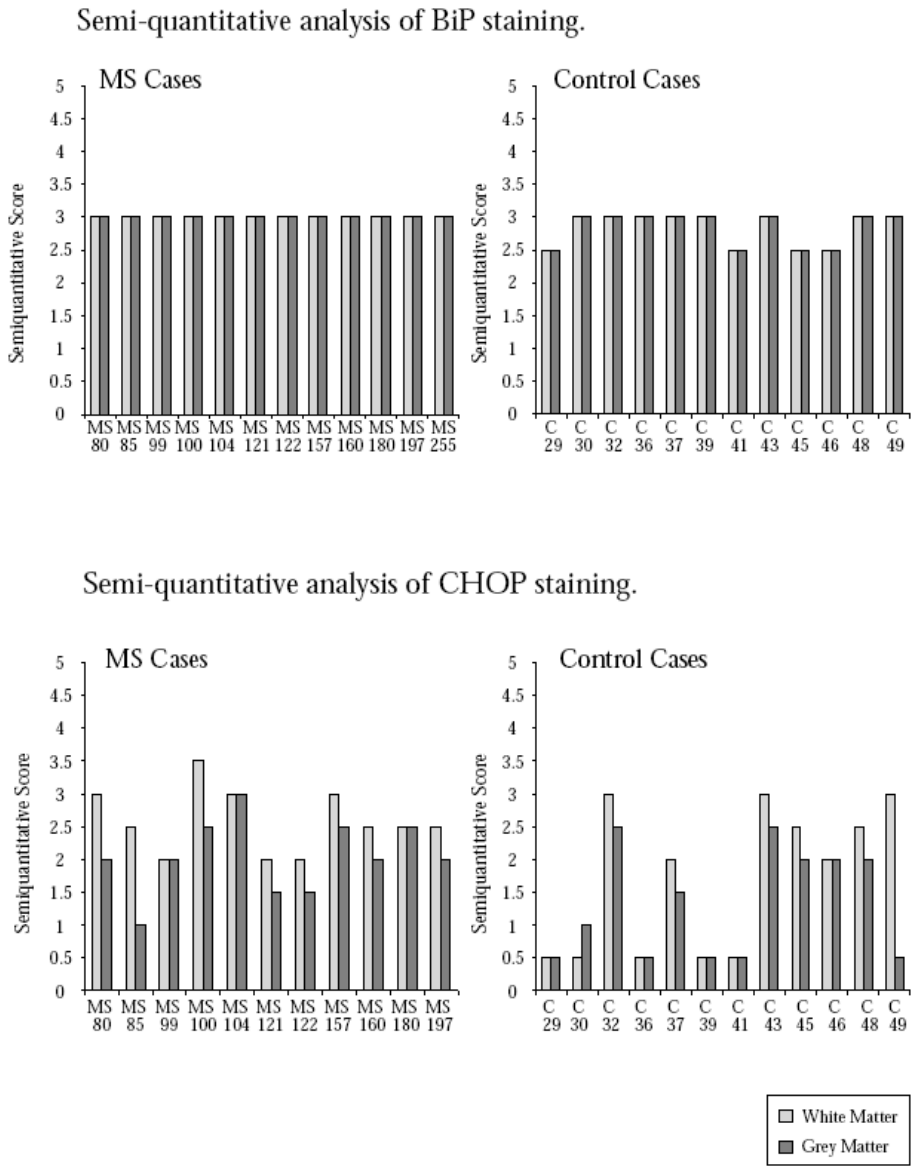

Figure 5. Semi-quantitative analysis of BiP and CHOP-staining in GM & WM areas of MS and control non-MS cases.

BiP staining was generally at a high level of expression in the majority of sections analysed, and little difference was seen between MS cases, with GM pathology, and non-MS controls (Figures 5 & 6).The majority of positive cells had morphology in keeping with neurones (Figure 6). Because of this high expression level in both groups no further analysis of BiP staining was carried out. Analysis on NAGM slides stained with BiP and CHOP has not yet been completed. D110-stained slides from all categories are still being analysed.

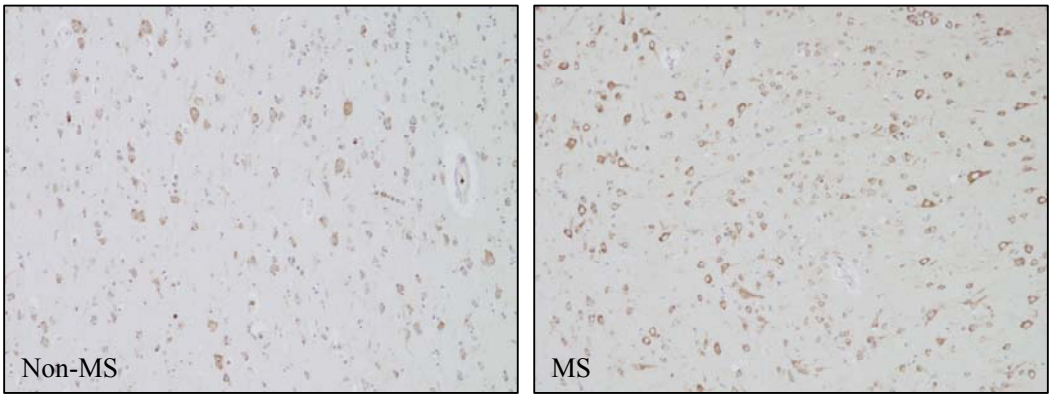

Figure 6. BiP staining in MS case and control non-MS

Analysis of CHOP staining indicated that there seemed to be a trend towards raised levels of CHOP staining in MS cases compared to non-MS controls (Figure 5). Further analysis was carried out to try and determine levels of CHOP positive cells in and adjacent to GM lesions. Counts were carried out as described in Methodology section.

Overall there did not seem to be any correlation between the numbers of CHOP-positive cells and areas of demyelination. In general, the majority of Type I and Type III lesions showed small number of positive cells in comparison to WM lesions and Type II lesions (Figure 7).

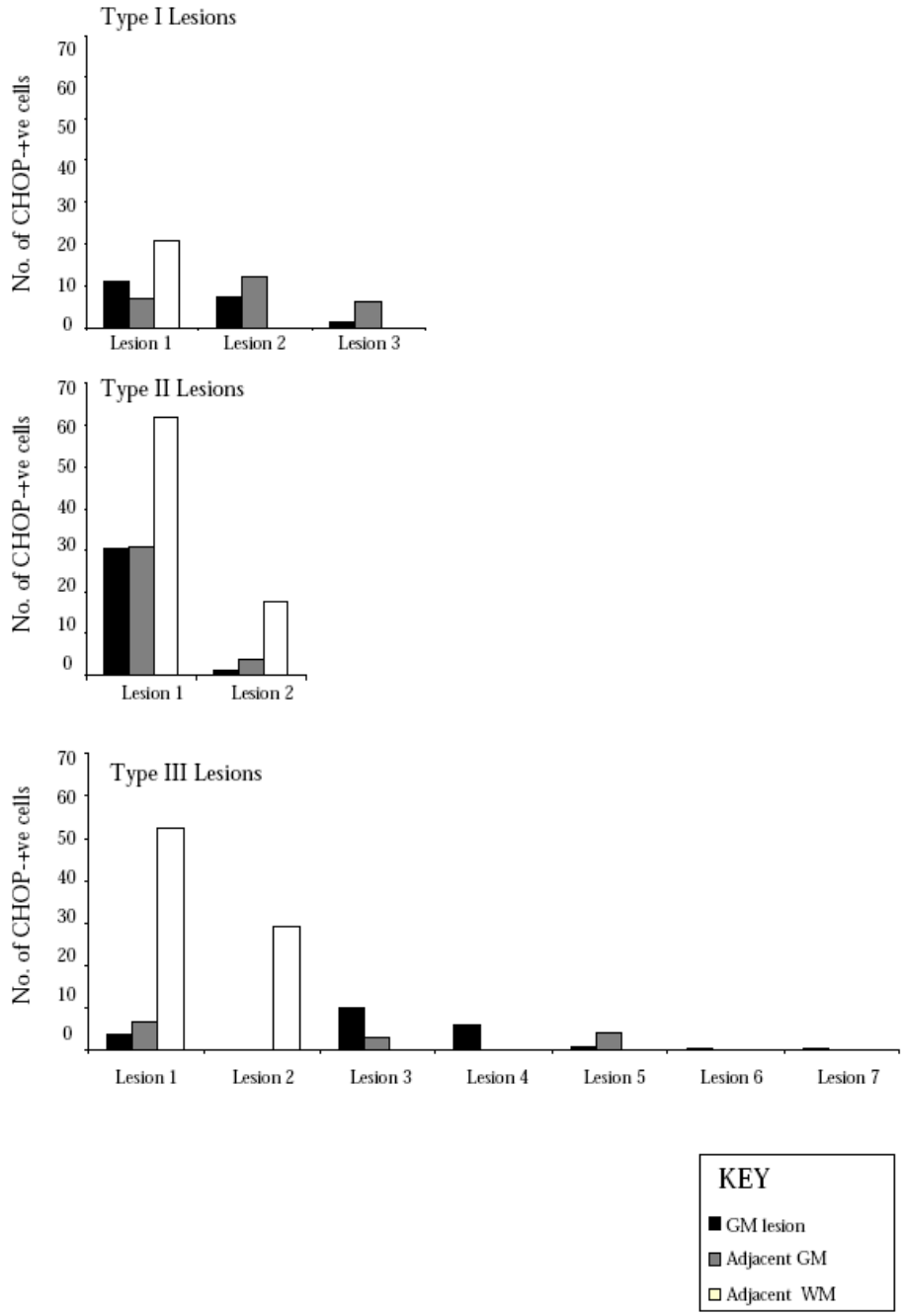

Figure 7. Quantitation of numbers of CHOP-+ve cells in MS GM lesions and in adjacent GM and WM areas.

Although CHOP positivity was seen in a variety of cell types (having morphology consistent with those of glial cells and neurones), there was a noteworthy number of positive cells having microglial morphology, in areas adjacent to GM lesions (Figure 8).

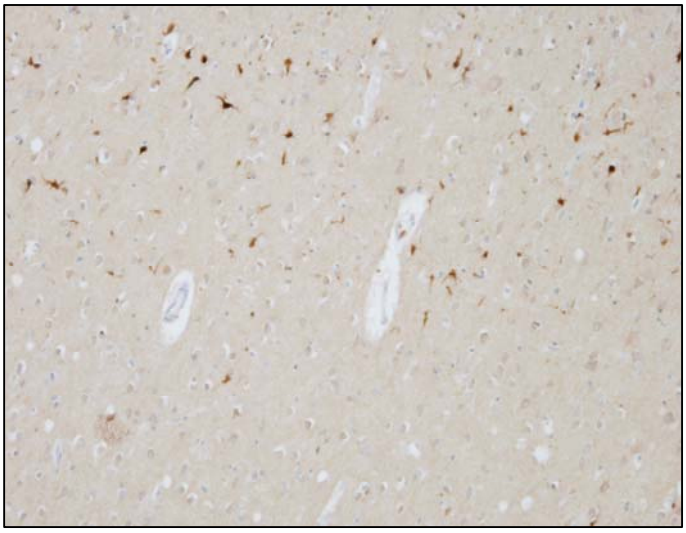

Figure 8. CHOP positive cells (stained dark brown) in a grey matter area adjacent to GM demyelination. These cells have a morphology in keeping with that of microglia.

CHOP +ve cells do not seem to have the same distribution in GM lesions as they do in WM lesions. Many different cells types in, and adjacent to, GM lesions, showed a degree of positivity including neurones and glia. The predominant cell type (as identified by morphological criteria) most often showing CHOP positivity was microglia, and often these positive cells were seen adjacent to lesions rather than within an area of grey matter demyelination.

The reason for upregulation of CHOP within microglial cells remains unknown. It is possible that the quiescent resident microglia require activation of ER-stress pathways in order to differentiate into the more mobile "ameboid" type of cell which may be involved in innate type immune responses brought about by a perturbed environment in and around the area of grey matter demyelination. It has been previously reported that macrophages upregulate ER-stress-associated molecules on activation and differentiation, so it is reasonable to propose that a similar mechanism may occur in microglia. The exact role of these immune cells, and their reasons for upregulating CHOP in areas of GM pathology remains unknown, but further research into the nature of these responses could provide some very interesting insights.

Neurodegeneration

SMI 32 and RT 97 immunostaining was carried out in order to see if neurodegeneration was evident within GM lesions and whether this could be correlated with ER-stress associated molecules. Initial evaluation of these slides at 4x magnification indicated that there was no obvious change seen in neurofilaments seen in association either with areas of ER-stress molecule positivity or with GM lesions. However, it is possible that further analysis could be carried out using stereology and axon width measurements to detect neurodegenerative change.

A subset of slides, showing strong CHOP positivity, was stained for βAPP which is known to be an indicator of axonal damage. While β-APP positivity was seen in some WM areas, there was no evidence of positive cells in either normal-appearing or lesioned GM material.

In summary, this project has provided us with a lot of material which can help towards answering some questions regarding GM pathology in secondary progressive MS. That the GM lesions show an inherently different pattern from the WM lesions is not surprising, as it is well documented that the respective types of pathology are different. However, it is very interesting that ER-stress associated molecules may play a role in grey matter demyelination, perhaps at a "pre-lesional" stage, raising some very interesting questions as to what is actually occurring prior to and during this much "quieter" type of pathology. Once all results are collected from the stained slides, this study can be used in conjunction with other studies within our lab (ex vivo de- and re-myelination studies and the analyses of grey matter pathology in an animal model) to allow further hypotheses to be generated regarding the roles played by ER stress and hypoxia**.[1, 29]**

8. Recommendations for practise, research and for broader policy issues

This ongoing research project into ER-stress involvement in grey matter pathology is one that is an integral part of a multi-faceted, comprehensive study looking at the molecular events occurring in MS pathology. This overall study involves a range of source material including human brain tissue (biopsy and post-mortem tissue), human CSF, *ex vivo* de- and re-myelination models, as well as two animal models of MS. These EAE (experimental autoimmune encephalomyslitis)

models of MS, induced in rats, result in two very different types of pathology being seen. “Classic EAE”results in widespread demyelination in spinal cord whereas the “Grey Matter EAE” model which results in cortical demyelination. These different human animal and tissue studies allow a comprehensive investigation to be made both into white matter and grey matter demyelination and how ER stress and/or hypoxia may feature in disease pathology.

In the current project, a specific emphasis was placed on grey matter demyelination in secondary progressive cases of MS, using formalin-fixed paraffin-embedded post-mortem tissue. Although the results seen differ from those we have previously observed in WM pathology, they still raise interesting questions regarding the cellular and molecular events occurring during grey matter lesion formation and contribute greatly to the overall picture of ER-stress involvement in MS pathogenesis.

Further investigation into this subject is recommended in order to determine the role being played in microglia by ER-stress associated molecules in, and adjacent to, areas of cortical demyelination. Once this can be elucidated, and once it can be established whether the responses are predominantly protective or pro-apoptotic, a better understanding of the events leading to grey matter demyelination will be gained. Understanding of such pathways, and the events leading to “life-or-death” decisions being made at the cellular level, may provide the basis for novel therapeutic agents.

9. Bibliography

1. Bo, L., et al., *Subpial demyelination in the cerebral cortex of multiple sclerosis patients.* J Neuropathol Exp Neurol, 2003. **62**(7): p. 723-32.
2. Kutzelnigg, A., et al., *Widespread demyelination in the cerebellar cortex in multiple sclerosis.* Brain Pathol, 2007. **17**(1): p. 38-44.
3. Magliozzi, R., et al., *Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology.* Brain, 2007. **130**(Pt 4): p. 1089-104.
4. Peterson, J.W., et al., *Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions.* Ann Neurol, 2001. **50**(3): p. 389-400.

5. Vercellino, M., et al., *Grey matter pathology in multiple sclerosis.* J Neuropathol Exp Neurol, 2005. **64**(12): p. 1101-7.
6. Bo, L., et al., *Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration.* Mult Scler, 2003. **9**(4): p. 323-31.
7. Brink, B.P., et al., *The pathology of multiple sclerosis is location-dependent: no significant complement activation is detected in purely cortical lesions.* J Neuropathol Exp Neurol, 2005. **64**(2): p. 147-55.
8. Dutta, R., et al., *Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients.* Ann Neurol, 2006. **59**(3): p. 478-89.
9. Diaz-Sanchez, M., et al., *Protein co-expression with axonal injury in multiple sclerosis plaques.* Acta Neuropathol, 2006. **111**(4): p. 289-99.
10. Hoozemans, J.J., et al., *Activation of the unfolded protein response in Parkinson's disease.* Biochem Biophys Res Commun, 2007. **354**(3): p. 707-11.
11. Hoozemans, J.J., et al., *The unfolded protein response is activated in Alzheimer's disease.* Acta Neuropathol, 2005. **110**(2): p. 165-72.
12. Oh, Y.K., et al., *Superoxide dismutase 1 mutants related to amyotrophic lateral sclerosis induce endoplasmic stress in neuro2a cells.* J Neurochem, 2008. **104**(4): p. 993-1005.
13. Marciniak, S.J., et al., *CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum.* Genes Dev, 2004. **18**(24): p. 3066-77.
14. McCullough, K.D., et al., *Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state.* Mol Cell Biol, 2001. **21**(4): p. 1249-59.
15. Yamaguchi, H. and H.G. Wang, *CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells.* J Biol Chem, 2004. **279**(44): p. 45495-502.
16. NiMhaille, A., et al., *Increased expression of endoplasmic reticulum stress-related signaling pathway molecules in multiple sclerosis lesions.* J Neuropathol Exp Neurol, 2008. **67**(3): p. 200-11.
17. NiMhaille, A., et al., *Endoplasmic reticulum stress: a valid therapeutic target in multiple sclerosis?* . Ann Neurol, 2010. **(submitted)**.

18. Xu, W., et al., *Nitric oxide induces coupling of mitochondrial signalling with the endoplasmic reticulum stress response.* Nat Cell Biol, 2004. **6**(11): p. 1129-34.
19. Gass, J.N., et al., *Stressed-out B cells? Plasma-cell differentiation and the unfolded protein response.* Trends Immunol, 2004. **25**(1): p. 17-24.
20. Rodriguez, M. and B. Scheithauer, *Ultrastructure of multiple sclerosis.* Ultrastruct Pathol, 1994. **18**(1-2): p. 3-13.
21. Bo, L., *The histopathology of grey matter demyelination in multiple sclerosis.* Acta Neurol Scand Suppl, 2009(189): p. 51-7.
22. Itoyama, Y., et al., *Immunocytochemical method to identify myelin basic protein in oligodendroglia and myelin sheaths of the human nervous system.* Ann Neurol, 1980. **7**(2): p. 157-66.
23. Hasegawa, M., et al., *Development of myelination in the human fetal and infant cerebrum: a myelin basic protein immunohistochemical study.* Brain Dev, 1992. **14**(1): p. 1-6.
24. Geurts, J.J., et al., *Intracortical lesions in multiple sclerosis: improved detection with 3D double inversion-recovery MR imaging.* Radiology, 2005. **236**(1): p. 254-60.
25. Cercignani, M., et al., *Magnetisation transfer ratio and mean diffusivity of normal appearing white and grey matter from patients with multiple sclerosis.* J Neurol Neurosurg Psychiatry, 2001. **70**(3): p. 311-7.
26. Rovaris, M., et al., *Short-term accrual of gray matter pathology in patients with progressive multiple sclerosis: an in vivo study using diffusion tensor MRI.* Neuroimage, 2005. **24**(4): p. 1139-46.
27. Barkhof, F., *The clinico-radiological paradox in multiple sclerosis revisited.* Curr Opin Neurol, 2002. **15**(3): p. 239-45.
28. Kutzelnigg, A., et al., *Cortical demyelination and diffuse white matter injury in multiple sclerosis.* Brain, 2005. **128**(Pt 11): p. 2705-12.
29. Geurts, J.J., et al., *Gray matter pathology in (chronic) MS: modern views on an early observation.* J Neurol Sci, 2009. **282**(1-2): p. 12-20.
30. Martinon, F., et al., *TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages.* Nat Immunol. **11**(5): p. 411-8.
31. Kidd, D., et al., *Cortical lesions in multiple sclerosis.* Brain, 1999. **122 (Pt 1)**: p. 17-26.