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ENDOPLASMIC RETICULUM STRESS
SIGNALLING IN MYELINATION

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A thesis submitted to the National University of Ireland, Galway in fulfilment of the requirement for the Degree of Doctor of Philosophy

July 2017
DECLARATION

I, the Candidate, certify that the Thesis is all my own work and that I have not obtained a degree in this University or elsewhere on the basis of any of this work.

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ABSTRACT

Demyelinating conditions such as multiple sclerosis have a continued unmet need for remyelinating therapies. Myelin is a multi-lamellar membrane structure made by oligodendrocyte progenitor cells (OPCs) that is critical for saltatory conduction and neuronal health. The simultaneous ensheathment of multiple axons with myelin confers a radically increased load on the endoplasmic reticulum (ER) from which membrane proteins and lipids are made. ER stress signalling triggers a conserved, homeostatic signalling system known as the Unfolded Protein Response (UPR) which can increase ER capacity or initiate apoptosis according to stress severity. Despite recognition of the substantial synthetic demands placed on oligodendrocytes, the role of ER stress signalling and the UPR has not previously been fully investigated. In this work, cerebellar tracts were used to exemplify the temporal dynamics of myelination. The cerebellum is an eloquent anatomical region of high clinical relevance to MS and a widely-used model of myelination, however the majority of studies do not take into account the well-established heterogeneity of its parasagittal domains. Therefore this work has combined a comprehensive profile of ER stress and UPR-associated markers with respect to the developing and adult cerebellum in the cortex as well as in white matter tracts. It then investigated the function of these pathways during oligodendrocyte differentiation in vitro. UPR signalling was found to be unaltered between parasagittal domains of the cerebellum, suggesting it is not an intrinsic factor in patterned neurodegeneration as previously indicated. Distinct UPR profiles were associated with specific cell-types during development and highlighted transient, proliferative subpopulations in germinal niches of the cerebellum. Actively myelinating cerebellar tracts showed selective upregulation of ATF6 and IRE1 signalling in the absence of PERK activation. In addition, high basal expression of ATF6 and ER-resident chaperones was observed in mature oligodendrocytes, indicating the usage of a nuanced UPR. Modulators of UPR signalling were tested on differentiating OPCs in vitro and impairments to oligodendrocyte maturation were observed. Changes in myelin gene expression, however, did not correlate with UPR markers. This work supports the activation of the UPR during myelination and highlights how approaches to the study of the UPR may be challenging in current models. This work draws attention to how myelin synthesis is facilitated by the ER of oligodendrocytes which merits further study.
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CHAPTER 1

INTRODUCTION
1.1 MULTIPLE SCLEROSIS

1.1.1 Introduction

Multiple Sclerosis (MS) is a chronic and debilitating neurodegenerative autoimmune disease with no current cure. MS is estimated to affect 2.3 million people worldwide although incidence rates vary according to sex, genetics and environment (MSIF, 2013; Browne et al., 2014) (Fig. 1.1). It is the most common chronic neurological disorder in young adults, usually presenting between 15-45 years of age. MS has major impacts physically, mentally and socioeconomically, including higher rates of mortality, comorbidities and is one of the leading causes of paralysis (Capkun et al., 2015; Multiple Sclerosis Ireland, 2015; Armour et al., 2016). Life span for people with MS is ca. 10 years shorter than age-matched controls (Brønnum-Hansen, Koch-Henriksen and Stenager, 2004). Ireland is within the region of highest risk, with an average prevalence of 140 per 100,000 (Lonergan et al., 2011) and an annual incidence rate of 5.97 new cases per 100,000 (MSIF, 2013; O’Connell et al., 2015).

Figure 1.1. Prevalence of MS varies greatly worldwide. Image from (Browne et al., 2014).

MS is termed a demyelinating disorder because lesions, also known as ‘plaques’, occur in the central nervous system (CNS) where there is an absence of myelin. These plaques are easily detectable by magnetic resonance imaging (MRI) in the
white matter (i.e. myelinated axonal tracts) but are less obvious in the grey matter where myelin is sparser (e.g. cortex and inner nuclei of the brain and the central spinal cord). Grey matter damage is usually only observed by more sensitive immunohistochemical methods that are impossible in the living patient but this is changing thanks to technological developments such as double inversion recovery techniques and high field Tesla MRI (Gilmore et al., 2009; Zivadinov and Pirko, 2012). Brain atrophy is present from the onset of MS and correlates with subsequent disability status (Chard et al., 2002; Fisher et al., 2002; Bergsland et al., 2012). It is a distinguishing feature of MS that is not observed in neuromyelitis optica (NMO), another demyelinating auto-immune disorder, once commonly confused with MS (Matthews et al., 2015). The auto-immune target of NMO is an astrocyte-specific protein called aquaporin-4 whereas no common antigen has been identified in MS.

Though not a hereditary disease, there are genetic factors that can make a person at greater risk of developing MS (International Multiple Sclerosis Genetics Consortium et al., 2011). A meta-analysis of twin studies indicated that heritability was responsible for 50% of MS liability variance, the twins’ shared environment for 21%, and the unshared environment for 29% (Fagnani et al., 2015). A female bias is a common trait of auto-immune diseases and similarly two thirds of people with MS are women (MSIF, 2013; Ngo, Steyn and McCombe, 2014). The incidence of MS is highly variable across the globe but a consistent gradient is observed between latitudes, with those furthest from the equator carrying higher MS risk. More specifically, sun exposure in early life may strongly determine a person’s risk of MS. A wealth of studies now point towards a protective role of vitamin D in reducing MS risk which may partly explain this distribution (Hanwell and Banwell, 2011). In contrast, MS risk is increased by smoking and people who are seropositive for the Epstein Barr virus are 15 times more likely to contract MS (Ascherio and Munger, 2007a, 2007b). EBV has been linked with reactivation of endogenous retroviruses as a potential driver of disease (Morandi et al., 2017).
1.1.2 MS diagnosis and classification

Unlike NMO, MS lesions may occur anywhere in the CNS, leading to diverse symptoms according to the functional significance of the damaged area (Matthews et al., 2013). Some of the most common symptoms include fatigue, pain, problems with movement, sensation, coordination, vision, urinary and bowel issues, sexual dysfunction, cognition and mood. The majority of lesions detected on MRI are asymptomatic (Thorpe et al., 1996). Lesions can be repaired and re-occur in the same and different regions over time, “dissemination in time and space” being an essential hallmark of MS. While patients can sometimes sense an impending attack, treatment management is nonetheless very difficult, and available therapies are limited and expensive. Diagnosis of MS is made through clinical and/or paraclinical assessments using the McDonald criteria (Polman et al., 2011). Dissemination in space criteria is met by existence of lesions in more than one CNS region. Dissemination in time is determined by a new lesion with respect to a baseline scan or the presence of a gadolinium-enhancing lesion and a non-enhancing lesion at the same time. In addition, the presence of oligoclonal bands or an elevated IgG index in cerebrospinal fluid (CSF) is strongly supportive of a clinical diagnosis of MS.

- Clinically Isolated Syndrome

A person experiencing a first episode of CNS inflammation or demyelination may recover and never contract a relapse. Such is the case for 80% of people presenting with a normal MRI scan, and for 20% of those with an abnormal MRI scan (Brex et al., 2002). This is termed Clinically Isolated Syndrome (CIS) and is the default designation before a relapse can confirm a clinical diagnosis of MS. Until then, it is unknown who will develop MS, although those presenting with optic neuritis are more likely to be associated with a more positive outcome than those presenting with cerebellar symptoms (Kantarci, Wingerchukb and Wingerchuk, 2006). Radiologically Isolated Syndrome (RIS) represents the case of an incidental finding of a lesion by MRI suggestive of demyelination which is clinically silent (Lublin et al., 2014). Although MS can have an unpredictable course, over time most patients are stratified into four main categories (World Health Organization, 2006; Lublin et al., 2014).
- **Benign MS**

Benign MS is a controversial term that has been defined in various ways, e.g. normal employment though not necessarily symptom-free after 10 years, an Expanded Disability Status Scale (EDSS (Kurtzke, 1983)) score of < 3 after ten years, or deterioration of not more than one grade of disability in 5 years. Despite varying definitions it is thought that up to 30% experience a benign course and some estimate that subclinical MS may even be as common as MS itself (Ramsaransing and De Keyser, 2006, 2007). However, such a diagnosis can only be assigned retrospectively (Lublin *et al.*, 2014).

- **Relapsing Remitting (RRMS)**

Of those diagnosed with MS, 85% experience multiple acute episodes lasting from days to months, followed by a spontaneous, full or partial recovery.

- **Secondary Progressive (SPMS)**

Within 20 years from diagnosis, 60-80% of people with RRMS develop SPMS, after which functional recovery does not return to baseline and progressive disability accumulates (MSIF, 2013; Scalfari *et al.*, 2014).

- **Primary Progressive (PPMS)**

For 10-15% of people with MS, rather than presenting as acute and episodic with periods of remission, their symptoms are chronic, stationary and worsen over time. Onset is typically later than in RRMS, in the 40-50s age range. Some also experience relapses and may be described as progressive relapsing.

At present there are no prognostic tests available to determine whether someone will go on to develop MS after an initial attack, or which type of disease course they will have. Some features of CIS and early MS can be indicative of severity of disease course (Table 1.1).
Table 1.1 Features of CIS and early MS indicative of severity of disease course

<table>
<thead>
<tr>
<th>More positive prognosis</th>
<th>Poorer prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger age of onset</td>
<td>Older age of onset (&gt;40 years)</td>
</tr>
<tr>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Onset with optic neuritis or isolated</td>
<td>Multifocal onset or cerebellar, motor or efferent systems affected</td>
</tr>
<tr>
<td>sensory symptoms</td>
<td></td>
</tr>
<tr>
<td>Full recovery from relapse</td>
<td>Partial recovery</td>
</tr>
<tr>
<td>Long interval to second attack</td>
<td>High relapse rate in first 2-5 years</td>
</tr>
<tr>
<td>No disability after 5 years</td>
<td>Substantial disability after 5 years</td>
</tr>
<tr>
<td>Normal MRI/low lesion load</td>
<td>Abnormal MRI/high lesion load</td>
</tr>
<tr>
<td>No posterior fossa lesions</td>
<td>Posterior fossa lesions</td>
</tr>
</tbody>
</table>

(Hawkins and McDonnell, 1999; Miller et al., 2005; Scalfari et al., 2014).

1.1.3 MS pathology
Multiple Sclerosis is so-called because it leaves behind multiple plaques of demyelination that are overlaid with firm, “sclerotic”, glial scar tissue over time (Cruveilhier, 1842). As with many neurological disorders, the ultimate lack of access to affected tissue until post-mortem, long after disease inception, has hampered efforts to understand the aetiology of MS. The histopathology of MS lesions was most richly described by James Dawson but it continues to yield new insights to the present day (Fig. 1.2) (Dawson, 1916; Jürgens et al., 2016). Features of actively demyelinating lesions in the WM include reduced myelin density and irregular ensheathment of axons in the presence of infiltrating lymphocytes and activated microglia/macrophages containing phagocytosed myelin. Active lesions are believed to progress to either chronic active (“smouldering plaques” where demyelinating activity is limited to the lesion edge), inactive (chronically demyelinated regions without inflammatory infiltrate) or remyelinated lesions (“shadow plaques” with characteristically shorter internodes and thinner myelin sheaths than in adjacent normal-appearing regions). Examples of all categories may be seen in the same patient. Histopathological analysis of 120 cases from acute MS, RRMS, PPMS and SPMS (with or without relapses) revealed two thirds of all lesions in RRMS to be active. In contrast, the same proportion of lesions were found to be inactive in SPMS without relapses. Chronic active lesions were a feature of SPMS and PPMS only. After >1 year, the proportion of remyelinated lesions did not appear to change with
disease duration, though the timing of remyelination cannot be known due to the vulnerability of such lesions to re-attack over time (Frischer et al., 2015).

Figure 1.2 Histopathology of multiple sclerosis in white matter and grey matter lesions described by J.W. Dawson. Figures 16-17 show sharply demarcated demyelination with preservation of axons evidenced by Bielschowski’s stain. Figures 18-20 demonstrate the presence of “Gitter” fat granule cells in active lesions, likely to be phagocytic macrophages containing myelin. Figures 21-22 illustrate glial changes in the demyelinated cortex (Dawson, 1916).

Although cortical pathology in MS was recognised early on (Fig. 1.2), its relevance was dismissed for many decades. Demyelinated lesions in the cortex cannot be
determined by routine MRI but can be present from onset (Lucchinetti et al., 2011). They typically exhibit little lymphocytic infiltrate and are instead currently classified by region selectivity. (Bø et al., 2003; Prins et al., 2015). Type 1 lesions (leukocortical) extend across GM and the sub-cortical WM; type 2 lesions (intracortical) are contained entirely within the cortex and do not touch the pia mater or sub-cortical WM; type 3 lesions (sub-pial) extend from the pia mater into the cortex but do not contact the WM and are the most frequently observed GM lesion; type 4 lesions span the entire width of the cortex but do not enter the WM. Cortical demyelination is especially pronounced in the cerebellum with an average of ~40% of the total area affected in SPMS, but can be as high as 83-92% (Kutzelnigg et al., 2007; Howell et al., 2015). Demyelination also occurs in GM structures such as the thalamus, basal ganglia, hippocampus and spinal cord GM. As such, total GM lesion load can exceed that of WM in terms of percentage area affected (Gilmore et al., 2009; Geurts et al., 2012).

Cortical pathology is not limited to demyelination. Loss of dendritic spines, neuron cell body atrophy, axon injury and ultimate neuron and axon loss occur in the presence and absence of cortical demyelination (DeLuca et al., 2006; Klaver et al., 2015; Jürgens et al., 2016). Although neuronal loss can occur as a result of WM damage far from the site of the cell body, it is now accepted that neurodegeneration in MS cannot be explained by demyelination alone (Friese, Schattling and Fugger, 2014). Inflammation and lymphoid-like structures in the meninges may be critical drivers of GM pathology and neuronal loss in progressive MS (Serafini et al., 2004; Magliozzi et al., 2007, 2010; Howell et al., 2011). Research in this field is rapidly advancing in an effort to understand neurodegeneration and the disability that accrues in the apparent absence of relapses.

A landmark study by Lassmann, et al. attempted to discern pathogenic differences from biopsies and post-mortem cases of acute MS (Lassmann, Brück and Lucchinetti, 2001). Actively demyelinating lesions were assigned to four different subtypes. Type I described sharply demarcated, inflammatory lesions, typically surrounding a central vein, containing infiltrating T cells and macrophages. In addition to these features, Type II exhibited prominent complement and immunoglobulin (Ig) deposition which was not observed in other subtypes. Type III lesions also contained inflammatory cells but they were not associated with a central
blood vessel. The edges were ill-defined and frequently emerged as alternating rings resembling Balo’s scleroses. Oligodendrocytes were absent in the lesion core and apoptotic at the lesion edge. Type IV lesions were distinguished by the presence of dying oligodendrocytes in white matter preceding the area of myelin destruction and were observed only in some cases of primary progressive disease. As each case of MS consistently presented with active lesions of a single subtype, it was postulated that each could indicate a distinct pathogenesis of disease. Thence types I and II were associated with the classical autoimmune hypothesis while type III and IV were ascribed to dysfunctional or dystrophic oligodendrocytes. Debate continues on this classification system. Some claim, for instance, that type III lesions as so-described are in fact common to most MS cases and represent a very early stage of newly forming lesions (Barnett and Prineas, 2004).

Given the wide variation in clinical presentation, disease course and histopathology, MS, as currently defined, may represent more than one disease. There is strong evidence for the classical auto-immune hypothesis of MS. Oligoclonal bands detected from the cerebrospinal fluid of patients indicate local production of Igs in the CNS (Quintana et al., 2012). The strongest genetic associations for MS also implicate the immune system, particularly MHC class II HLA-DRB1 risk alleles (Broadley et al., 2000; Lock et al., 2002; DeLuca et al., 2007; International Multiple Sclerosis Genetics Consortium et al., 2011). Therapies blocking immune cell infiltration are effective in reducing relapse rate. Similarly to other auto-immune diseases, incidence shows a female bias (MSIF, 2013; Ngo, Steyn and McCombe, 2014). No common autoantigen has been identified, however, and the same autoantibodies have been detected in both MS and non-MS subjects. The majority of disease-modifying therapies (DMTs) that show efficacy for MS have no benefit in the defined autoimmune CNS disorder NMO and can instead induce severe exacerbations of disease (Kira, 2017). It is still argued whether MS truly originates from an auto-immune disease or whether, due to reasons unknown, oligodendrocyte cell death occurs initially and consequent myelin debris, inflammation and microglial/macrophage activation follows (Lucchinetti et al., 2000; Trapp and Nave, 2008; Nakahara et al., 2012). Regardless of the initial insult, release of antigens and upregulation of major histocompatibility complex (MHC) molecules from an inflammatory CNS environment may be sufficient to drive an immune response.
Released antigens from CNS myelin proteins or cross-reactive pathogens could prime T and B cells in peripheral lymph nodes. After stimulation and clonal expansion, lymphocytic infiltration across the blood brain barrier (BBB) could cause direct damage to MHC class I presenting cells, initiate production of IgG antibodies by plasma cells, and recruit further microglial activation and macrophages to the lesion (Hemmer, Archelos and Hartung, 2002). Interestingly, transgenic mice which had remyelinated and recovered from induced oligodendrocyte ablation by diphtheria toxin, were found to develop a fatal, secondary, demyelinating disease characterised by CD4$^+$ T cell-mediated auto-immunity against myelin about 30 weeks later (Traka et al., 2010, 2015).

1.1.4 Current treatments – Unmet needs
The number of treatments for MS has expanded greatly in the past twenty years. The range of available therapies exhibit modest, moderate or high efficacy but come with parallel increases in risk profiles. Current treatments largely help to prolong remission between attacks and so are beneficial for RRMS patients only. DMTs showing modest effects, such as interferon beta and glatiramer acetate, modulate the immune response and can reduce relapse rate and delay CIS to MS conversion (The IFNB Multiple Sclerosis Study Group., 1993; Johnson et al., 1995; Jacobs et al., 1996; Wingerchuk and Weinshenker, 2016). However accumulated disability is not directly proportional to number of relapses. Concomitant factors of the inflammatory disease and aging are believed to be more important factors. Higher performing DMTs such as daclizumab (a monoclonal antibody against CD25 expressed on activated T cells) and fingolimod (sphingosine-1-phosphate receptor (S1P) inhibitor that prevents egress of lymphocytes from lymph nodes) perform better than interferon beta when measured by relapse rate and MRI activity but have a higher adverse event profile. Natalizumab (a monoclonal antibody against $\alpha$4 integrin which inhibits transmigration of leukocytes out of the vasculature) has the highest efficacy of DMTs regarding relapse rate, disability progression and brain atrophy but leaves patients vulnerable to a frequently fatal viral disease called progressive multifocal leukoencephalopathy (PML). PML is caused by reactivation of the ubiquitous John Cunningham virus. Although this virus generally has no effect on non-immunosuppressed people, it can lie dormant in the CNS and cause aggressive
and rapid demyelination upon reactivation. Patients seropositive for the virus have higher incidence of PML (1:132 vs <1:10,000) but this risk accumulates with time and increases, for those with prior immunosuppressive therapy, to as high as 1:31 after 24 months of therapy (Schwab et al., 2017).

Patients with progressive MS receive no benefit from most current treatments. Ocrelizumab (a monoclonal antibody against CD20 expressed on B cells) has very recently become the first FDA-approved treatment for PPMS. Promisingly, an S1P modulator, siponimod, has also shown efficacy against disability progression in a phase III clinical trial for SPMS (NCT01665144).

Autologous haemopoietic stem-cell transplantation (HSCT) following aggressive chemotherapy and immunosuppression has yielded unprecedented cessation of disease activity up to 13 years after treatment (Atkins et al., 2016). This unparalleled efficacy also carries the greatest risk profile. Of 24 recruited patients. One person died and another was admitted to intensive care due to complications of sinusoid obstruction syndrome. All experienced febrile neutropenia and over 25% experienced secondary autoimmunity and viral infections.

Clearly there is a need for effective therapies that do not compromise on safety, and for approaches that extend beyond solely targeting immune cells. This approach focuses on only one aspect of a still misunderstood, multi-factorial disease and largely benefits only a proportion of the RRMS disease phenotype. Attention is only recently being drawn to myelin restoration, neuroprotection and oligodendrocyte-protective therapies which may be beneficial to both demyelinating and neurodegenerative disorders alike (Franklin et al., 2012; Kremer et al., 2016; Tognatta and Miller, 2016; Verden and Macklin, 2016).

1.2 MYELINATION

1.2.1 Myelin

Myelin is the result of concentric rolls of membrane extruded from oligodendrocytes being wrapped around an axon. It bears a fatty, white, glistening appearance that gives rise to the white and grey matter delineations of the CNS. In humans, white matter accounts for ~ 50% of brain volume and contains 118,000 km of myelinated
fibres in the forebrain alone (Tang and Nyengaard, 1997; Zhang and Sejnowski, 2000). The grey matter is also extensively myelinated in a protracted development that continues into late adulthood (Miller et al., 2012; Haroutunian et al., 2014).

This significant investment by the CNS yields high velocity conduction of action potentials. This occurs because potassium (K⁺) ion channels become concentrated at nodal regions of the axonal membrane. These nodes of Ranvier demarcate the endpoints of the myelin sheath where the axon is relatively exposed and devoid of basal lamina. By confining the area of depolarization to these pockets of excitable axolemma, the impulse jumps from node to node in a manner known as saltatory conduction. This reduces the sodium flux entering the nerve, conserves energy, reduces axon calibre and speeds the rate of impulse conduction dramatically. For example, a myelinated nerve in a frog conducts at 25 m/s at 20°C and has a diameter of 12 µm. The unmyelinated giant axon of the squid achieves the same velocity, but is 500 µm in diameter, uses 5,000 times as much energy and occupies about 1,500 times as much space (Quarles, Macklin and Morell, 2006). In addition myelin shows plastic properties similar to neuronal plasticity. Node number, length and thickness can alter in response to neuronal activity, learning, social changes and even gut bacteria, as well as disease (Bengtsson et al., 2005; Scholz et al., 2009; Hu et al., 2011; Liu et al., 2012; Makinodan et al., 2012; Gibson et al., 2014; McKenzie et al., 2014; Hoban et al., 2016).

Beyond enhancement of conduction properties, myelin mediates biochemical metabolic coupling and exosome transfer between oligodendrocytes and neurons (Fünfschilling et al., 2012; Frühbeis et al., 2013; Simons and Nave, 2015). Monocarboxylate transporters MCT1 and MCT2 are expressed on myelin and axons respectively and evidence suggests that they mediate transfer of glycolytic products across the myelin membrane to support the energy demands of axons (Rinholm et al., 2011; Lee et al., 2012). In MS, denuded, energy-deficient axons attempt to compensate by increasing the number of mitochondria. When remyelinated, this number is reduced, though it does not return to normal levels (Zambonin et al., 2011). Myelin supports axonal integrity, not only by shielding axons, but also through specific axo-glial interactions. Changes in the molecular composition in myelin have been shown to cause neuronal degeneration even when these changes do not compromise myelin stability (Griffiths et al., 1998; Garbern et al., 2002; Lappe-
Multiple system atrophy, a relentless neurodegenerative disorder affecting the autonomic nervous system, cerebellum, substantia nigra and striatum, is first and foremost “a primary oligodendrogiopathy” (Jellinger, 2014). Myelin is hypothesised to play a protective role in other neurodegenerative diseases such as Parkinson’s disease in which only poorly myelinated and unmyelinated axons are vulnerable (Braak and Del Tredici, 2004a, 2015; Braak et al., 2004b; Orimo et al., 2011).

This new appreciation of myelin functions has come with increased understanding of its composition and complexity. The myelin proteome has been completely revised in recent years with the advent of advanced mass spectrometry methods (De Monasterio-Schrader et al., 2012). In addition the myelin lipidome has been more extensively characterised (Gopalakrishnan et al., 2013) and a unique myelin transcriptome has been identified for the distal synthesis of membrane components during myelin turnover (Thakurela et al., 2016).

Myelin is believed to have first evolved to facilitate rapid escape and predatory behaviours (Zalc, 2016). It is now implicated in diverse spectra of disease. Beyond primary demyelinating diseases such as MS, NMO and hereditary leukodystrophies, myelin aberrations are also observed in schizophrenia, autism, neurodevelopmental and neurodegenerative disorders (Fields, 2008; Haroutunian et al., 2014; Kremer et al., 2016). Although remyelination is imperfect, the breadth of functional benefits afforded to myelinated axons suggest that promoting myelination in MS could be neuroprotective, prevent axonal loss and associated disability, and may have potentially far-reaching benefits for other neurological disorders also (Franklin et al., 2012; Braak and Del Tredici, 2015; Kremer et al., 2016).

1.2.2 Oligodendrocytes

Oligodendrocytes are the myelin-ensheathing cells of the CNS. They evolved independently from Schwann cells, their counterparts in the PNS. They differ in myelin composition and node to cell ratio although some myelin constituents are common to both. In the PNS, Schwann cells rotate around a single axon as they wrap it in layers of membrane. These compact to form a single myelin segment enveloped by cytoplasm on the inner and outer layers. In contrast, oligodendrocytes extend and retract processes before establishing axonal contact and initiate wrapping of multiple
axons simultaneously. This process was only recently revealed to follow a “croissant” style in which the inner tongue advances under the previous wrap of membrane (Snaidero et al., 2014). Radiating cytoplasmic (“myelinitic”) channels deliver vesicles to support the growth of the leading edge during development. In mature myelin, cytoplasm is restricted to the outer lip, adaxonal layer, and paranodal loops but the myelinitic channels can reopen upon stimulation (Snaidero et al., 2014). Myelin formation occurs rapidly, within 5 h of initiation in zebrafish and 12-18 h in rat (Watkins et al., 2008; Czopka, Ffrench-Constant and Lyons, 2013). Depending on node number and axon diameter, different sources have estimated the relative membrane surface area of myelin to an oligodendrocyte cell body as approximately 1:300-6000 (Pfeiffer, Warrington and Bansal, 1993), 1:620-3,000 (Brady et al., 2005, p. 14) or expressed as a rate of approximately 1:16-166 per day during active myelination (Baron and Hoekstra, 2010). In any case, the construction of this unique structure is a colossal feat which must then be maintained throughout life. Once myelination is initiated, cells do not recover this ability to form myelin segments and a significant fraction die during developmental myelination (Barres et al., 1992; Keirstead and Blakemore, 1997; Trapp et al., 1997).

The differentiation of oligodendrocytes is, therefore, highly regulated. Based on transcriptome analysis, up to 12 distinct subpopulations can be identified spanning the continuum of the oligodendrocyte lineage in vivo (Marques et al., 2016). In practice, three broader subtypes are used to categorise oligodendrocyte stages of maturation; oligodendrocyte progenitor cells (OPCs), premyelinating oligodendrocytes and mature oligodendrocytes (Fig. 1.3).
OPCs are mitotically-active and highly motile. They arise from several sources in the CNS with distinct transcriptional control mechanisms (Goldman and Kuypers, 2015). Two waves of ventrally-derived OPCs from the medial and lateral ganglionic eminences come to populate the forebrain but these are largely displaced by a third wave around birth from the dorsal subventricular zone (Kessaris et al., 2006). Similarly, two distinct waves emanate from the ventral and dorsal spinal cord but in this case the ventral OPCs predominate. The majority of oligodendrocytes in the cerebellum originate from ventral rhombomere 1, with ca. 6% originating from the
cerebellar subventricular zone (Hashimoto et al., 2016). OPCs from different origins can ultimately replace each other and show no differences in electrical properties (Kessaris et al., 2006; Tripathi et al., 2011). However, dorsally-derived oligodendrocytes were shown to dominate in remyelinated lesions after LPC was administered in the corpus callosum and ventral spinal cord and exhibited different functional profiles than ventral OPCs in response to aging (Crawford et al., 2016).

As OPCs differentiate into pre-myelinating oligodendrocytes, they lose expression of early markers A2B5, NG2 and PDGFRα and can be identified by the O4 antibody which binds to an unidentified sulfatide (Fig. 1.3). They are less migratory, become post-mitotic and bear multiple processes that initiate contact with axons. The terminal differentiation into mature oligodendrocytes is confirmed by expression of myelin proteins such as PLP, MBP and MOG. Mature OL cell bodies can also be identified by APC/CC1 or GSTpi antibodies. Caspr is an axonal protein specifically expressed at paranodes and can be another useful confirmation of myelin formation.

1.2.3 Myelin synthesis

In comparison to plasma membrane, the composition of myelin has a higher lipid to protein ratio which is roughly 70:30 in dry weight terms. The lipid composition of myelin changes during development. At first it is higher in phospholipids, similar to plasma membrane, and then the proportion of cholesterol and glycosphingolipids increases until a stable molar ratio is reached of 4:4:2 - 4:2:2 for cholesterol, phospholipids and glycolipids, respectively (Cuzner and Davison, 1968; Norton and Poduslo, 1973; Baumann and Pham-Dinh, 2001; Ozgen et al., 2016). The brain accounts for approximately 25% of all cholesterol in the body, 80% of which is in myelin. Virtually all CNS cholesterol is generated by de novo local synthesis as the BBB blocks lipid exchange from the circulation (Björkhem and Meaney, 2004). Myelin is specifically enriched with the sphingolipids galactosylerceramide (GalC) and sulfatide which are compartmentalised in compact and non-compact myelin respectively (Ozgen et al., 2016). The synthesis of all these lipid components is initiated at the endoplasmic reticulum (ER) (Fig. 1.4) (Blom, Somerharju and Ikonen, 2011).
Figure 1.4. Biosynthetic pathways of major membrane lipids are initiated at the endoplasmic reticulum. The synthesis for phospholipids (left), sphingolipids (middle) and cholesterol (right) are all initiated at the ER. Figure from (Blom, Somerharju and Ikonen, 2011).

Myelin proteins are also highly compartmentalised in the myelin sheath. The relative abundance of myelin proteins, as determined by mass spectrometry, demonstrates PLP to be the most abundant (17%), followed by MBP (8%), CNP (4%) and MOG and others at 1% or less (De Monasterio-Schrader et al., 2012). Due to its specific function in compaction, mRNAs of MBP are trafficked to the end of oligodendrocyte processes and translated locally to ensure controlled compaction of membranes (Laursen, Chan and FfrenchConstant, 2011). PLP and MOG, like the majority of membrane proteins, are synthesised by ribosomes at the ER.

Ultrastructural studies of CNS development have repeatedly shown that as OPCs transition to actively myelinating cells, their ER profiles increase to form large cisternae in ordered parallel stacks, in contrast to the short scattered cisternae of early oligodendrocytes, and they then become less prominent in fully mature cells.
Despite this, the central role of the ER in the synthesis of myelin components has received little attention.

1.3 THE ENDOPLASMIC RETICULUM

1.3.1 Introduction

As the principle site of synthesis for lipids and proteins, the endoplasmic reticulum is the fulcrum for change in cells. It is involved in growth, expansion, differentiation, calcium signalling and homeostasis, and more specialised functions according to cell type. The endoplasmic reticulum (“little net within the cytoplasm”) is a dynamic organelle highly interconnected to the cytoskeletal framework. As a microtubular mesh, the ER is able to interact with the cytoskeleton and rearrange via tubule elongation, retraction and sliding, branching and anastomosis (Lee and Chen, 1988; Friedman et al., 2010). According to its sub-cellular position, function and cell type, it may form flattened sacs, cisternal sheets, linear tubules, polygonal reticula and three-way junctions (Lee and Chen, 1988; Dreier and Rapoport, 2000). Present in all eukaryotic cell types except for mature erythrocytes, ova, embryonic and prokaryotic cells, it is especially prominent in neurons, hepatocytes, adreno-cortical cells, retinal cells, myocytes, interstitial cells and steroid-synthesising cells.

The ER closely associates with all other subcellular organelles, i.e. the Golgi, plasma membrane, mitochondria, vacuoles, peroxisomes, endosomes and lysosomes as well as ribosomes. Its functions are equally far reaching. It is the synthetic hub for lipids, membranous and secreted proteins, and is also responsible for their correct folding and post-translational modifications such as glycosylation and the addition of disulfide bonds. It is also necessary for xenobiotic metabolism, synthesis of phospholipids and steroids, membrane and ER biogenesis, storage and release of cellular calcium. The ER is also considered an excitable signalling system, capable of transmitting calcium both as an input and output (Berridge, 2002).

A common luminal space is shared between the nuclear envelope and throughout the reticular formations of the ER, as demonstrated in cerebellar Purkinje neurons (Terasaki et al., 1994; Dayel, Hom and Verkman, 1999). The ER can be subdivided
into the nuclear envelope, rough ER and smooth ER. The rough ER is often more tubular, granular and is studded with ribosomes whilst the smooth ER is more elaborate, convoluted and expansive (Baumann and Walz, 2001; Voeltz, Rolls and Rapoport, 2002). Through its close association with ribosomes, the primary function of the rough ER is protein synthesis and glycosylation. The smooth ER is the site of phospholipid and fatty acid synthesis. It is prominent in certain cell types where it is functionally specific, such as neurons, hepatocytes, adreno-cortical cells, retinal cells, interstitial cells and steroid-synthesising cells. It is responsible for antibody secretion in leukocytes and the production of insulin in pancreatic cells. In the liver, enzymes in the smooth ER detoxify hydrophobic toxins, while in muscle it is known as the sarcoplasmic reticulum where it acts as a calcium release store for muscle contraction. In the brain, the smooth ER is required for the synthesis of hormones and is crucial for calcium signalling throughout the dendrites and axons of neurons. A transitional ER is also proposed for the packaging of proteins for transport to the Golgi (Palade, 1975; Hobman et al., 1998).

Most transmembrane and secreted proteins are first synthesised on the ER surface as unfolded polypeptide chains and enter the specialised, calcium-rich, oxidizing environment of the ER lumen to undergo post-translational modifications and guided structural collapse to adopt their correct final conformations. Strict quality control mechanisms are necessary to prevent misfolded proteins from exiting the ER and threatening the function and viability of the cell. To ensure high fidelity, ER-resident chaperone proteins occupy various sites of nascent proteins to ensure they fold correctly. Once this process is successful, a molecular tag allows the protein to exit the ER lumen. However, if a protein continually fails to achieve the correct conformation, it will be targeted by the endoplasmic reticulum associated protein degradation pathway (ERAD) (Meusser et al., 2005).

The ER must be dynamic and adaptable to meet changing demands whether due to infection, injury, or due to physiological changes in cell cycling, nutrient status or other environmental changes. Homeostatic control is maintained via three transmembrane sensors that detect internal changes on their luminal surface and translate to effectors on their cytosolic side. These sensors are protein kinase RNA-like endoplasmic reticulum kinase (also known as eukaryotic translation initiation factor 2-alpha kinase 3, pancreatic ER eIF2α kinase, or PERK), activating
transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). Increases in misfolded or unfolded proteins in the ER lumen elicit the Unfolded Protein Response (UPR), an evolutionarily conserved response that can determine cell fate. Deletion of a single UPR initiator or signalling protein is often sufficient to cause embryonic or perinatal lethality (Table 1.2). The UPR was first discovered when an accumulation of unfolded proteins in the ER were observed to induce the specific transcriptional upregulation of ER resident chaperone proteins (Kozutsumi et al., 1988).

### Table 1.2: Phenotype of knockout mice for UPR signalling proteins.

<table>
<thead>
<tr>
<th>Knockout</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UPR initiators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERK</td>
<td>Perinatal lethality, type I diabetes, bone abnormalities</td>
<td>(Harding et al., 2001)</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Embryonic lethal</td>
<td>(Zhang et al., 2005)</td>
</tr>
<tr>
<td>IRE1β</td>
<td>Viable. Increased ER stress in goblet cells</td>
<td>(Tsuru et al., 2013)</td>
</tr>
<tr>
<td>ATF6α</td>
<td>Phenotypically normal</td>
<td>(Yamamoto et al., 2007)</td>
</tr>
<tr>
<td>ATF6β</td>
<td>Phenotypically normal</td>
<td>(Yamamoto et al., 2007)</td>
</tr>
<tr>
<td>ATF6α/ATF6β</td>
<td>Embryonic lethal</td>
<td>(Yamamoto et al., 2007)</td>
</tr>
<tr>
<td><strong>UPR mediators and targets</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eIF2α</td>
<td>Perinatal death, diabetes, pancreatic β-cell deficiency</td>
<td>(Scheuner et al., 2001)</td>
</tr>
<tr>
<td>ATF4</td>
<td>Embryonic/perinatal lethality</td>
<td>(Tanaka et al., 1998)</td>
</tr>
<tr>
<td>GADD34</td>
<td>Phenotypically normal</td>
<td>(Kojima et al., 2003)</td>
</tr>
<tr>
<td>CHOP</td>
<td>Phenotypically normal</td>
<td>(Zinszner et al., 1998)</td>
</tr>
<tr>
<td>XBP1</td>
<td>Embryonic lethal</td>
<td>(Reimold et al., 2000)</td>
</tr>
<tr>
<td>GRP78</td>
<td>Embryonic lethal</td>
<td>(Luo et al., 2006)</td>
</tr>
<tr>
<td>GRP94</td>
<td>Embryonic lethal</td>
<td>(Wanderling et al., 2007; Yang et al., 2007)</td>
</tr>
<tr>
<td>PDIA1</td>
<td>No viable strain yet reported</td>
<td>(Galligan and Petersen, 2012)</td>
</tr>
</tbody>
</table>

Various disruptions to homeostasis can lead to compromised efficiency and the accumulation of misfolded proteins, i.e. ER stress, including nutrient deprivation,
calcium ion imbalance, increased translational demand or ERAD defects. The 78 kDa glucose-regulated protein, GRP78 (also known as HSPA5, binding immunoglobulin protein or BiP), is a HSP70 chaperone which binds to nascent proteins as they enter the ER and to sites on the luminal domains of PERK, ATF6 and IRE1. In the classical model of UPR activation, GRP78 is bound to ER stress sensors during homeostatic conditions and preferentially binds to unfolded proteins as they accumulate. The dissociation of GRP78 from ER stress sensors acts as a common trigger to initiate a three-pronged response (Fig. 1.5).

**Figure 1.5.** Summary of signalling pathways of the Unfolded Protein Response. ER stress is detected on the luminal side of stress sensors (A). Signalling intermediaries modulate transcriptional and translational outputs of the cell via transcription factors ATF4, XBP1s, ATF6(N) (B). The net result of UPR signalling can determine cell survival or apoptosis in the face of ER stress (C). Image source: M. Naughton.

### 1.3.2 PERK pathway

The most rapidly activated arm of the UPR in response to ER stress is that initiated by PERK. The cytoplasmic protein kinase domain undergoes trans-
autophosphorylation and phosphorylates eukaryotic translation initiation factor 2 α (eIF2α) (Fig. 1.6). In addition to activated PERK, eIF2α can be phosphorylated by three other similar kinases that each respond to distinct environmental stimuli; general control non-derepressible 2 (GCN2) in response to amino acid deprivation, double-stranded RNA-dependent protein kinase (PKR) in response to viral infection, or heme-regulated eIF2α kinase (HRI) in response to heme deprivation (Pakos-Zebrucka et al., 2016). The convergence of these signals on peIF2α is called the Integrated Stress Response (ISR). peIF2α in turn inhibits eIF2B and the assembly of 80S ribosomes, with the net result of reducing the protein load entering the ER by attenuating global protein translation. Select mRNAs are preferentially translated however. Such mRNAs contain multiple upstream open reading frames (ORFs) in their 5’ UTR, which can only be bypassed under conditions of eIF2α phosphorylation. The most well-documented of these is activating transcription factor 4 (ATF4) which encodes a cAMP response element-binding transcription factor. Approximately half of all PERK-dependent UPR target genes are ATF4-dependent, including genes that aid amino acid metabolism, redox regulation and protein secretion (Harding et al., 2003). Endogenous ATF4 is rapidly targeted for proteasomal degradation, with a half-life of 30 minutes under non-stressed conditions (Lassot et al., 2001; Rutkowski et al., 2006). During pronounced or prolonged ER stress, the transcription factor C/EBP homologous protein (CHOP, also known as DNA Damage-Inducible Transcript 3 protein, DDIT3, and Growth Arrest and DNA Damage-inducible protein, GADD153) is induced. Compared with ATF4, CHOP mRNA has a single upstream ORF and other conserved RNA elements that are sufficient to inhibit translation during homeostasis but when eIF2α is phosphorylated, scanning ribosomes are able to bypass the upstream ORF and initiate translation at the CHOP coding region (Palam, Baird and Wek, 2011). Likewise CHOP mRNA and protein are rapidly degraded (2-4 h) and as such CHOP levels strongly reflect current levels of stress (Rutkowski et al., 2006). CHOP suppresses Bcl2, induces Bim and DR5, promotes caspase activation and is pro-apoptotic in many cell types (Mccullough et al., 2001; Silva et al., 2005; Song et al., 2008; Li et al., 2009; Y.-H. Zhang et al., 2011; Carlisle et al., 2014), though another of its targets, GADD34, may more strongly correlate to ER stress-induced cell death(Rutkowski et al., 2006). Recently, miR216b has been identified as a CHOP-
dependent miRNA that targets c-Jun and further sensitizes cells to ER stress-induced apoptosis (Xu et al., 2016). In addition, the PERK/peIF2α pathway also promotes autophagosome formation which is increasingly linked to degradation of proteins as well as bulk cellular contents as part of the cellular defence system (Kouroku et al., 2006).

**Figure 1.6.** PERK signalling arm of the Unfolded Protein Response. Activated PERK induces a translation block via phosphorylation of eIF2α. The transcription factor ATF4 is preferentially expressed under such conditions, one of select mRNAs that can circumvent this blockade. It upregulates GADD34 which de-phosphorylates eIF2α and allows resumption of translation. The transcription factor CHOP is upregulated during severe or prolonged stress and is classically associated with ER stress-induced apoptosis. Image source: M. Naughton.

### 1.3.3 ATF6 pathway

ATF6 is a diffusible, membrane-bound, UPR-specific transcription factor in mammals (Haze et al., 1999; Shen et al., 2005). It lies in the ER lumen as an inactive precursor. When GRP78 dissociates, ATF6 transits to the Golgi to undergo proteolytic cleavage by site 1 and site 2 proteases (Fig. 1.7) (Ye et al., 2000). This releases the active cytosolic transcription factor which moves to the nucleus where it upregulates genes involved in ER protein folding, secretion and degradation. The ATF6-GRP78 complex appears relatively stable and diffuses freely in the ER lumen.
In the case of ATF6, there is evidence against the passive competition model and that, instead, an active mechanism triggers GRP78 dissociation and consequent ATF6 transit (Shen et al., 2005).

**Figure 1.7.** ATF6 signalling arm of the Unfolded Protein Response. During ER stress ATF6 transits to the Golgi apparatus where it is cleaved by site-1 and site-2 proteases. The active transcription factor potently upregulates genes for increasing ER capacity including chaperone proteins, folding enzymes and ER membrane biogenesis. Image source: M. Naughton.

### 1.3.4 IRE1 pathway

IRE1 is both a kinase and an endoribonuclease. It oligomerizes and trans-autophosphorylates after GRP78 dissociation (Fig. 1.8). The conformational change induced in its cytosolic domain allows binding to the adaptor protein tumour-necrosis factor-α (TNFα)-receptor-associated factor 2, known as TRAF2. This leads to activation of the Jun N-terminal kinase (JNK) pathway and the nuclear translocation of NFκB via IkB kinase, and subsequent production of TNFα. Upregulation of inflammatory cytokines, and activation of pro-apoptotic B cell lymphoma 2 (Bcl2) family members via JNK, shift cell fate towards apoptosis. The best characterised substrate for IRE1’s endoribonuclease activity is X-box binding protein 1 (XBP1) from which a 26 base intron is removed. When translated, spliced XBP1(XBP1s) reveals an active bZIP-family transcription factor (Rutkowski and Kaufman, 2007). XBP1s can form homo- and hetero-dimers with ATF6 and ATF4.
and upregulates ERAD factors and chaperones, thus increasing the capacity of the ER for processing and folding nascent proteins (Schröder and Kaufman, 2005).

**Figure 1.8.** IRE1 signalling arm of the Unfolded Protein Response. Activated IRE1 can activate the JNK pathway via TRAF2 binding and shift cell fate to apoptosis. It also splices XBP1 mRNA to facilitate translation of a potent transcription factor for ER expansion and folding proteins however. In addition, activated IRE1 degrades ER-localised mRNAs during ER stress through RIDD. Image source: M. Naughton.

In addition, regulated IRE1-dependent decay (RIDD) is an important mechanism for regulating ER protein load by degrading ER-localised mRNA and miRNA transcripts. RIDD substrates, which include ER-resident chaperones GRP78 and PDI, are cleaved at an XBP1-like consensus site and are rapidly degraded by cellular exoribonucleases. This activity increases proportionally with ER stress duration and intensity and promotes ER stress-induced apoptosis (Han et al., 2009; Maurel et al., 2014). Alternate modes of IRE1 activation induced by kinase inhibitors such as APY29 can favour XBP1 splicing activity and avert RNA decay and apoptosis (Han et al., 2009). In contrast, salicyladehyde-based inhibitors such as 4μ8C can block XBP1 splicing and RIDD activity but do not affect IRE1 phosphorylation (Han et al., 2009; Maly and Papa, 2014).

In summary, ER stress occurs when the protein folding capacity of the ER fails to meet demand and unfolded proteins accumulate. Dissociation of GRP78 from transmembrane sensors on the ER lumen triggers the UPR. In acute stress, signal
transduction via PERK rapidly inhibits protein translation so that more proteins do not continue to overload the ER. PERK activation is rapidly reversed upon restoration of homeostasis. If stress is prolonged, longer term adaptations are made via transcriptional upregulation of genes targeting ER resident chaperones, enzymes, ER biogenesis and ERAD. If this fails; ER stress-induced apoptosis is triggered to safely dispose of the dysfunctional cell and its contents (Lin et al., 2007).

1.4 THE CEREBELLUM

1.4.1 The cerebellum as a model for study of CNS development and remyelination

Histological examination reveals a consistent laminar template throughout the cerebellum extending from the white matter to the pia via the granule cell, Purkinje cell (PC) and molecular layers. Although the cerebellum’s gross shape, volume and relative mass is altered considerably from rodent to man, this cytoarchitecture has remained remarkably conserved (J Voogd and Glickstein, 1998). Its stereotyped development and conserved modular circuitry has popularised the use of the cerebellum as a model for many CNS developmental processes. It has helped reveal fundamental aspects related to neurogenesis and circuit assembly (Van Welie, Smith and Watt, 2011; Butts, Green and Wingate, 2014). For instance, activity-dependent CNS circuit refinement is exemplified by the competitive elimination of surplus climbing fibres to innervate a single PC (Hashimoto and Kano, 2003, 2013).

The cerebellum has also been an important model for myelination, historically and up to the present day. Its laminar structure and predictable program of myelination facilitated early immunohistochemical and EM studies. This allowed in-depth analyses of the maturation program of myelinating oligodendrocytes in vivo (Ghandour et al., 1980; Hartman et al., 1982; Monge et al., 1986; Ghandour and Skoff, 1988; Reynolds and Wilkin, 1988; Friedman et al., 1989; Warrington and Pfeiffer, 1992; Levine, Stincone and Lee, 1993; Coffey and McDermott, 1997; Li, Hertzberg and Nagy, 1997). More complex analyses are now possible with newer methods and transgenic mice but cerebellar tissue sections continue to be exploited to help dissect signalling pathways important for myelination in vivo (e.g., Benninger et al., 2006; Zhao et al., 2007; Picou et al., 2012; Camacho et al., 2013).
Before the advent of IHC and modern assays, the cerebellum facilitated the use of organotypic CNS cultures for the study of myelinogenesis. The first such report by Hild was based on the roller tube method (Pomerat and Costero, 1956; Hild, 1957) and was expanded upon with further refinements of experimental *ex vivo* cerebellar models (Bornstein and Murray, 1958; Ross, Bornstein and Lehrer, 1962; Field, Hughes and Raine, 1969; Gähwiler *et al.*, 1997; Gähwiler, Thompson and Muller, 1999). These methods ultimately lead to substantial loss of 3D structure however and could be laborious and expensive (Jarjour *et al.*, 2012).

The current gold standard is the interface method using semi-porous membranes, which allows long-term culture without the excessive thinning associated with previous methods (Stoppini, Buchs and Muller, 1991). Prepared sagittal slices of cerebellum retains the extracellular matrix (ECM), three dimensional structure and intact projection neurons and spontaneously myelinate *ex vivo* (Fig. 1.9). This model has been embraced by the field for the study of oligodendrocyte biology both as acute slices (e.g., Bakiri *et al.*, 2011; Rinholm *et al.*, 2011; Garthwaite *et al.*, 2014) or as long-term organotypic cultures (Notterpek *et al.*, 1993; Ghoumari *et al.*, 2003; Miron *et al.*, 2010; Bin *et al.*, 2012; Sheridan and Dev, 2012; Hill *et al.*, 2013). Furthermore, cerebellar slices can be demyelinated by chemical or immune-mediated methods and subsequently exhibit the hallmarks of remyelination (i.e., shorter internodal lengths and larger G ratio) (Birgbauer, Rao and Webb, 2004; H. Zhang *et al.*, 2011). It has recently been used as an integral step in screens for potential remyelinating therapeutics (Najm *et al.*, 2015; Yao, Su and Verkman, 2016).
Many underpinnings of CNS development and myelination are grounded in studies utilising the cerebellum. It is furthermore a region highly relevant to MS pathology (section 1.4.12) (Weier et al., 2015). In order to best interpret previously published data and use this model for new investigations, the gross and cellular organisation of the cerebellum and its development is reviewed.

1.4.1 Gross anatomy of the cerebellum

The cerebellum, or “little brain”, is present in all vertebrates, occupying the space behind the 4th ventricle. Two lateral hemispheres converge on a central region called the vermis. Like the cerebrum, the cerebellum possesses a folded cortex overlying white matter tracts that convey to inner nuclei, but it takes up only one tenth of brain volume (Ekinci et al., 2008). Although compact, it holds 80% of all CNS neurons (Azevedo et al., 2009; Herculano-Houzel, 2009).

The cerebellum is split into the anterior lobe and posterior lobe by the primary fissure. The flocculonodular lobes are separated from the posterior lobe by posterolateral fissures at either side. Transverse fissures further divide the cerebellum into lobules (10 in rat, Fig. 1.10), which can be grouped into transverse zones based on gene expression; the anterior zone (vermal lobules I-V and paravermal extensions), central zone (lobules VI-VII), posterior zone (VIII-dorsal IX) and nodular zone (ventral IX and X) (Larsell, 1952; Apps and Hawkes, 2009).
1.4.2 Laminar organisation of the cerebellum

Histology reveals a consistent laminar template throughout the cerebellum (Fig. 1.11). Purkinje cells (PCs) are arranged in a uniform monolayer in the cerebellar cortex. Beneath the Purkinje cell layer (PCL) is the granule cell layer (GL) which contains billions of excitatory granule cell neurons. Peripheral to the PCL is the molecular layer (ML) which is sparsely populated by inhibitory interneurons amongst swathes of excitatory parallel fibres. The medullary or WM layer conveys efferent PC fibres to their targets in the deep cerebellar nuclei (DCN) and vestibular nuclei and supplies myelinated afferents of extracerebellar origin and the DCN to the cortex.
Figure 1.11. H&E/LFB staining shows the consistent laminar organisation of the cerebellum. (A) Low magnification of adult rat folium showing clear delineation of cortical layers. (B and C) Stratification of cerebellar tissue from WM to the pia mater at postnatal day 7 (B) and adult (C) at equivalent scale. Myelin is stained with luxol fast blue. Cortical layers are revealed by H&E staining. Abbreviations: egl, external germinal layer; gl, granule cell layer; ml, molecular layer; pcl, Purkinje cell layer; wm, white matter. Source: M. Naughton. Scale bars = 50μm.

1.4.3 Deep cerebellar nuclei

Cerebellar nuclei are absent in teleosts and first evolved as a single nucleus in each hemisphere in the elasmobranch (Ikenaga, Yoshida and Uematsu, 2006). Medial and lateral divisions giving rise to two nuclei are observed in dogfish, amphibians, reptiles, birds and marsupials (Altman and Bayer, 1996; Green and Wingate, 2014; Zhang, Wang and Zhu, 2016). Three distinct nuclei have evolved in mammals; fastigial, interposed and dentate. In primates, the interpositus nuclei are further split into the nucleus globosus and nucleus emboliformis and in man the dentate nucleus is increasingly elongated to the extent that it takes a convoluted “dentate” appearance.
1.4.4 Purkinje cells

Purkinje cells were first described by Johann Purkyne (Purkyne, 1838). The large pear-shaped somata (20-40μm) are arranged in a single “piriform” layer between the GL and ML (Fig. 1.12). They constitute the primary integrative unit and sole output of the cerebellar cortex. Their highly elaborate dendrites fan through the ML in a plane perpendicular to the pia. These receive excitatory inputs from parallel fibres and climbing fibres. Basket and stellate cells send inhibitory inputs to PC dendrites, soma and axon hillock. The PC axon becomes myelinated as it travels through the GL and gives off collaterals to synapse with basket cells, Golgi cells and other PCs. It breaks into a bushy terminal plexus and forms symmetrical en passant synapses with several neurons at its target nuclei. PCs from the vermis, paravermis and lateral hemispheres preferentially project to the ipsilateral fastigial, interpositus and dentate nuclei, respectively (Jan Voogd and Glickstein, 1998). Exceptions to this are PCs from the flocculonodular lobe which can project outside the cerebellum to the vestibular nuclei. PCs are GABA-ergic, therefore excitation of PCs inhibits the firing of their target neurons and inhibition of PCs facilitates the discharge of these neurons accordingly.

Figure 1.12. Purkinje cells possess pear-shaped somata that line the border between the granular layer and the molecular layer. GRP78 expression detected in Purkinje cell bodies and dendrites using DAB immunohistochemistry (brown). Source: M. Naughton. Scale bar = 50μm.
1.4.5 Excitatory neurons in the cerebellar cortex

Climbing fibres

Climbing fibres emerge from cell bodies in the inferior olive nucleus, which receives input from the spinal cord, brainstem, cerebellar nuclei and the motor cortex. Climbing fibres convey the cortico-pontine-cerebellar pathway, one of the largest tracts of the CNS, which is responsible for transmitting inputs from sensori-motor and visual association areas to the cerebellar cortex. Their myelinated axons are 1-3 μm in diameter and cross the midline at the level of the inferior olive in the medulla. They mainly enter the cerebellum via the inferior cerebellar peduncle, with a minor portion from the caudal part of the nucleus entering via the superior peduncle. Climbing fibres send collaterals to the deep cerebellar nuclei and branch into fine fibres within the white matter in a sagittal orientation. They pass through the dense GL unmyelinated and “climb” along Purkinje cell dendritic trees (Fig. 1.13).

Figure 1.13. A single climbing fibre (a) can form up to 300 synaptic contacts on one Purkinje cell (b), drawn by Ramon y Cajal (1911, p. 96 Fig. 22).

Approximately 10 climbing fibres emerge from a single olivary axon, each terminating on a single PC (Llinas, Walton and Lang, 2004). Climbing fibres are strongly excitatory, making up to 300 synaptic contacts along the soma, dendritic
trunk and main branches of a Purkinje cell neuron. A single pulse can elicit a short burst of action potentials from a PC and can reduce the effectiveness of local parallel fibres for some time.

*Mossy fibres*
Mossy fibres stem from vestibular, cerebellar and basilar pontine nuclei, as well as the spinal cord and reticular formation, and enter through all three cerebellar peduncles. They give off collaterals to the cerebellar nuclei, branch throughout the white matter transversally and synapse on granule cells. Mossy fibres entwine through the granule cells, enlarging at synaptic contacts to form “mossy fibre rosettes”, typically at the centre of glomeruli, through which a granule cell’s short “dendritic claws” can penetrate and synapse. A single mossy fibre may terminate in up to a dozen rosettes, each contacting up to 5 granule cells (Palkovits, Magyar and Szentagothai, 1972).

*Granule cells*
Granule cells are excitatory glutamatergic neurons and are by far the most numerous (and small) of CNS neurons, with a typical diameter of just 4-8μm (Llinas, Walton and Lang, 2004; Anttonen et al., 2005; Kalinichenko and Okhotin, 2005; Azevedo et al., 2009; Fitzgerald, Gruener and Mtui, 2012). They possess 3-5 short smooth dendrites which receive primary input from mossy fibres (Palkovits, Magyar and Szentagothai, 1972; Kalinichenko and Okhotin, 2005). Their axons are generally unmyelinated as they project past the PCL and perpendicularly bifurcate in the ML to form parallel fibres that extend several millimetres in both directions along the long axis of the folia. (4.2-4.7mm in the rat (Pichitpornchai, Rawson and Rees, 1994)). These parallel fibres are present at all levels of the ML and lie perpendicular to the plane of Purkinje cell dendritic fans. At intervals of ~3.7μm in the rat (Pichitpornchai, Rawson and Rees, 1994), they form a single *en passant* synapse with the peripheral branch of a PC dendrite that is crowned with spiny branchlets. While a parallel fibre might synapse with only one of every 3-4 PC fans that it passes, it can still synapse with up to 400 in total (Fitzgerald, Gruener and Mtui,
In this fashion, one PC tree receives excitatory input from up to 200,000 parallel fibres (Braitenberg and Atwood, 1958; Eccles, Ito and Szentágothai, 1967). No other neuron type receives such a huge number of inputs. The spacing and size of synaptic varicosities changes as the parallel fibre extends from its point of bifurcation, indicating that they may exert less influence on more lateral PCs relative to the granule cell body (Pichitpornchai, Rawson and Rees, 1994). Granule cells can also synapse with dendrites of basket cells, stellate cells and Golgi cells, and along the trunk of the PC, before it bifurcates.

The excitatory influence of mossy fibres on PCs is therefore indirect and spreads laterally over a large area of the cortex via parallel fibres. It would require the joint action of many parallel fibres to compete with the influence of a single climbing fibre but, as parallel fibres also make contact with interneurons of the cerebellar cortex, the mossy fibre pathway also has an inhibitory influence on PCs.

**Unipolar brush cells**

Unipolar brush cells are glutamatergic interneurons that excite granule cells in the GL (Kalinichenko and Okhotin, 2005). Their single thick dendrite ends in a “brush” of dendrioles that associates with mossy fibres (Fig. 1.14). In rodents UBCs are scarce in most lobules except lobules IX and X (vestibulocerebellum) but their distribution is more extensive in cerebella of carnivores and primates (Diño, Willard and Mugnaini, 1999). They are further concentrated within parasagittal zones (described further in section 1.4.9) (Diño, Willard and Mugnaini, 1999).

**1.4.6 Inhibitory interneurons in the cerebellar cortex**

**Golgi cells**

Golgi cells lie in the GL, are 6-11 μm in diameter, increase in size as they near the PCL (9-16 μm in diameter) and may represent a group of up to 5 subsets (Llinas, Walton and Lang, 2004; Schilling et al., 2008). They give off extensive radial dendrites that extend through all layers of the cerebellar cortex. As such, they receive input from mossy fibre, climbing fibre and PC collaterals, but they are mainly
contacted by excitatory synapses from parallel fibres. Their collateralised axons also branch in all directions (in contrast to PCs, basket and stellate cells).

**Figure 1.14.** Neuronal circuitry of the cerebellum. The two main types of afferents of the cerebellum are climbing fibres, which synapse directly with Purkinje cells, and mossy fibres, which branch transversely in the white matter and synapse with granule cells. Granule cell axons bifurcate in the molecular layer to form parallel fibres, which contact hundreds of Purkinje cell dendrites along the folia of cerebellar lobules. Cortical inputs and outputs are further regulated by interneurons including excitatory Golgi cells and unipolar brush cells, and inhibitory stellate and basket cells. Source: M. Naughton. Abbreviations: BC, basket cell; CF, climbing fibre; DCN, deep cerebellar nuclei; EC: extracerebellar origin; GC, granule cell; GL, granule cell layer; Go, Golgi cell; MF, mossy fibre; ML, molecular layer; SC, stellate cell; UBC, unipolar brush cell; WM, white matter.
They ramify extensively throughout the GL, surrounding climbing fibre rosettes and forming inhibitory synapses on granule cell dendrites. Two described subsets of Golgi cells are Lugaro cells and globular cells. Lugaro cell dendrites parallel to the PCL receive input from PC axon collaterals and serotonergic afferents. Their axons target interneurons (including Golgi cell dendrites) in the ML. Globular cell bodies reside in the upper GL but have dendrites and axons in the ML. They also target interneurons in the ML but their main input is not determined.

**Basket and stellate cells**

Basket and stellate cells belong to the one class and are both located in the ML where their processes extend sagittally to synapse with many cross-cutting parallel fibres. Their axons and dendrites are flattened, parallel to the long axis of the lobule. Stellate cells are found in the upper ML, decreasing in size closer to the periphery. Deeper stellate cells have more elaborate dendritic branching and can extend axons in parallel with the PC dendritic trees for up to 450 μm (Llinas, Walton and Lang, 2004). Basket cells lie closer to the PCL (Palay and Chan-Palay, 1974) and their dendrites can extend up to 300 μm. They are excited by presynaptic terminals of parallel fibres and their axons descend and turn at right angles to cross the PCL, forming *en passant* synapses with up to 150-250 PCs (Fitzgerald, Gruener and Mtui, 2012). Collaterals from as many as 50 different basket cells create a “weave” around the soma and initial segment of each Purkinje cell (Bishop, 1993). These form inhibitory axo-somatic synapses as well as unique *pinceaux* around the axon hillock which can block spontaneous and triggered PC firing and effectively regulate cerebellar output (Ramon y Cajal, 1911; Ito M., 1984).

**Candelabrum cells**

Candelabrum cells are also GABA-ergic and localise between PC somata in the PCL. They were first described in 1994 (Lainé and Axelrad, 1994) and many basic aspects remain ill-defined. Based on cell morphology, their likely afferents derive from the ML and their suggested targets include PCs and basket/stellate cell dendrites (Schilling *et al.*, 2008).
1.4.7 Cerebellar glia

Cerebellar glia perform similar functions as in other CNS regions but particular differences or adaptations are described below.

**Oligodendrocytes**

Until recently the primary source of cerebellar oligodendrocytes was unknown. Previous studies indicated that the majority of oligodendrocytes were of extracerebellar origin (Reynolds and Wilkin, 1988; Grimaldi et al., 2009). Fate-mapping analysis has determined that only 6% originate from the ventricular zone, and that the vast majority are derived from an Olig2-positive neuroepithelial domain in ventral rhombomere 1 at E11.5 in mouse and take approximately 5 days to reach the cerebellum (Hashimoto et al., 2016). Cerebellar oligodendrocytes are distributed throughout WM, DCN, GL and occur sparsely in the lower ML.

**Astrocytes**

Cerebellar astrocytes are derived from the ventricular zone (Buffo and Rossi, 2013). Classical fibrous and protoplasmic astrocytes are distributed throughout the adult WM and GL respectively while the ML contains specialised radial astrocytes called Bergmann glia (described below) (Fig. 1.15). Protoplasmic astrocytes are known to envelop glomeruli in the GL but do not penetrate them (Palay and Chan-Palay, 1974).

**Bergmann glia**

Bergmann glia are specialised astrocytes of the cerebellar cortex. Their cell bodies reside next to the PCL where they enfold PC somas and extend several ascending processes towards the pia, creating a palisade of glial fibres that are essential for granule cell migration during development (Fig. 1.15). They remain important for extracellular ion homeostasis, synaptic stability and plasticity into adulthood (De Zeeuw and Hoogland, 2015).

**Microglia**

Cerebellar microglia display different patterns of morphology and distribution according to laminar location (Fig. 1.16) (Vela et al., 1995; Perez-pouchoulen, Vanryzin and Mccarthy, 2015).
**Figure 1.15.** Astrocytes in adult cerebellum show distinct morphologies of fibrous astrocytes in WM, protoplasmic astrocytes in GL and Bergmann glia in PCL/ML. Astrocytes were detected by DAB immunohistochemistry (brown) with GFAP antibody and tissue was counterstained with hematoxylin (blue). Source: M. Naughton. Scale bar = 50μm.

**Figure 1.16.** Microglia in adult cerebellum stained with Iba1 display different morphologies according to tissue location. Panels from left to right show microglial cells in the GL, WM and ML. Microglia were detected by DAB immunohistochemistry (brown) with Iba1 antibody and tissue was counterstained with hematoxylin (blue). Source: M. Naughton. Scale bar = 20μm.
1.4.8 Cerebellar circuitry and function

The uniform cellular organisation of the cerebellum belies the breadth of functions it is known to effect. Far from the traditional view of the cerebellum being solely associated with motor function, functional connectivity MRI indicates that the cerebellar cortex contains multiple homotopic maps of the whole cerebrum (Fig. 1.17) (Buckner et al., 2011). This includes mirrored asymmetrical representations of areas involved with language and handedness (Wang, Buckner and Liu, 2013). The functional role of the cerebellum in higher cognitive tasks such as emotion, executive function, language, music, timing, and working memory is increasingly well-established (Buckner, 2013; E et al., 2014). Indeed grey matter density in contralateral frontal, parietal and temporal cortices was reduced after damage to the right cerebellar hemisphere and was associated with lower scores on executive function and memory tasks (Clausi et al., 2009).

![Figure 1.17](image-url)

**Figure 1.17.** Representation of cerebellar-cortical connectivity networks based on n=1000 subjects using resting state functional connectivity MRI (Buckner et al., 2011; Diedrichsen and Zotow, 2015). On the left is a flatmap representation of the human cerebellum showing the cerebral cortical resting-state network that correlated best with activity in that area. The corresponding region in the cerebral cortex is shown by matching number and colour on the right. Image source: (Diedrichsen and Zotow, 2015).
The first description of the cerebellar syndrome was only in terms of motor dysfunction but now a wide range of deficits associated with specific cerebellar regions are recognised (Table 1.3) (Bodranghien et al., 2016; Stoodley et al., 2016). The cerebellar motor syndrome is associated with anterior lobe and DCN damage and is characterised by impaired gait (ataxia), coordination (dysmetria), eye movements, articulation (dysarthria), swallowing (dysphagia), or tremor (Schmahmann, MacMore and Vangel, 2009). Cerebellar cognitive affective syndrome (CCAS) is now increasingly recognised since its description in 1998 (Schmahmann and Sherman, 1998). It is associated with damage to the posterior lobe and is characterised by impairments in executive functions (planning, abstract thinking, working memory, etc.), language (dysnomia, dysprodia, etc.) and mood disturbances (blunt affect to disinhibition and hyperexcitability).

The circuitry of the cerebellum can be broadly organised according to cortical region. The vermis and paravermis primarily receive sensory afferents from the spinal cord. PCs from the vermis project to the fastigial nucleus which innervates neurons in the brainstem and vestibular nuclei that project back to the spinal cord. This circuit is involved in the regulation of balance and control of voluntary movements. PCs from the paravermis project to the interposed and dentate nuclei, which indirectly innervate the motor cortex via the red nucleus and the thalamus. This is important for the control of limb movements and axial musculature.

PCs in the lateral hemispheres (neo- or cerebrocerebellum) receive input exclusively from the cerebral cortex via pontine nuclei. This input is in the form of mossy fibres which synapse on the contralateral cerebellar cortex. These PCs project to neurons in the dentate nucleus which transmit to the thalamus and red nucleus. Thalamic signals project back to the cerebral cortex. Neurons in the red nucleus project to the thalamus, spinal cord and the inferior olive, the latter resulting in innervation of the contralateral cerebellar hemisphere via climbing fibres.
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<td>Spatial span</td>
<td>Right cerebellum</td>
<td>15</td>
<td>VBM</td>
<td>(Clausi et al., 2009)</td>
</tr>
</tbody>
</table>
The flocculonodular lobe receives inputs from vestibular afferents and these PCs project back to the vestibular nuclei. In addition, the cerebellar nuclei and all three cortical layers receive neuromodulatory afferents (particularly serotonergic and dopaminergic) which may be important for cerebellar learning and control (Schweighofer, Doya and Kuroda, 2004).

1.4.9 Parasagittal molecular compartmentalisation of the cerebellum

The uniform cellular architecture of the cerebellum also masks a heterogeneous molecular profile that exhibits parasagittal topography. One of the first pieces of evidence of longitudinal compartmentalisation was observed in the white matter (Voogd, 1967). Haggqvist-stained transverse sections showed sharply delineated bands of myelinated fibres of different diameters. These bands corresponded to climbing fibres arising from specific inputs in the inferior olive (Armstrong, Harvey and Schild, 1974). The first molecule to display cerebellar “striping” was 5’ nucleotidase in 1963 (Scott, 1963) but the zebrin epitope, later identified as aldolase C, would become the most famous (Hawkes and Leclerc, 1987; Ahn et al., 1994). It revealed symmetrical, alternating bands of positive and negative PCs that span the medial-lateral axis. Further “striping” markers showed that banded expression aligned with the terminal fields of mossy fibre and climbing fibre inputs and could be further confined within transverse zones and sometimes even specific lobules (Díño, Willard and Mugnaini, 1999; Sugihara, Wu and Shinoda, 2001; Larouche and Hawkes, 2006; Demilly et al., 2011; Cerminara et al., 2015) (Fig. 1.18). Furthermore, banding complementarity can be reversed according to transverse zone as in the case of neurofilament H (Demilly et al., 2011) or may be temporary as in the case of calbindin during embryonic development (Larouche and Hawkes, 2006).
Accurate molecular patterning (but not foliation) is dependent on continued expression of engrailed homeobox genes *En1* and *En2* from as early as E9/10 in mouse (Sillitoe et al., 2008). A non-exhaustive list of molecules known to align with parasagittal bands includes: HSP25, excitatory amino acid transporter 4 (EAAT4), GABA B receptor subtype 2, neuronal calcium sensor 1, phospholipase Cβ3, phospholipase Cβ4, metabotropic glutamate receptor 1β (mGluR1β), microtubule-associated protein 1A, neuroplastin, neurogranin and neurofilament H (Armstrong et al., 2000; Chung, Kim and Hawkes, 2008; Demilly et al., 2011; Cerminara et al., 2015). These expression profiles carry functional effects on not just PCs but mossy fibres, climbing fibres, granule cells, ML interneurons and Bergmann glia. The compartmentalisation of the cerebellar cortex is also reflected in the DCN and projections of olivocerebellar neurons (Sugihara, Wu and Shinoda, 2001; Sugihara, 2011). Parasagittal bands influence patterns of electrophysiological activity, synaptic plasticity, long-term depression, excitotoxicity, neurodegeneration, neuroprotection after TBI and diverse other effects summarised by Cerminara et al., 2015 (Chen, Hanson and Ebner, 1996; Wadiche and Jahr, 2005; Gincel et al., 2007; Slemmer et al., 2007; Williams et al., 2007; Ebner et al., 2012; Hawkes, 2014; Zhou et al., 2014).

### 1.4.10 Cerebellar development

The cerebellum is one of the last brain regions to mature. It develops from the rostral lip of the 4th ventricle on the dorsal rhombencephalon at E13 in the rat but extensive
development occurs postnatally in the first three weeks after birth. Germinal niches associated with cerebellar development are the ventricular zone, rhombic lip, external germinal layer and the prospective white matter.

**Ventricular zone**
PCs and interneurons originate from the ventricular zone (the neuroepithelium of the fourth ventricle). PC clusters migrate to a differentiation zone and ascend to the PCL between embryonic day (E)17-21. By postnatal day (P)5, an even monolayer of PCs is formed beneath the ML. Apical swelling of PC bodies with sprouting processes is observed at P7 and at P10 a stem dendrite with multiple lateral branches is observed in the expanding ML. Interneuronal precursors, meanwhile, express cyclin D2 and Ki67 as they migrate through the prospective cerebellar white matter although they do not proliferate (Weisheit et al., 2006; Schilling et al., 2008). Their differentiation is staggered (e.g. Golgi cells mature before stellate cells) but nearly all reach their ultimate destinations by the end of the first postnatal week.

**Rhombic lip**
Granule cell precursors are produced in the external germinal layer which arises from the rhombic lip (described below). The rhombic lip also gives rise to secondary germinal zones, the rostral germinal zone and the anterolateral border with the cerebellar plate, in which UBCs and large projection DCN neurons are produced (Machold and Fishell, 2005; Englund et al., 2006; Marzban et al., 2015). Precursors of UBCs proliferate in the rhombic lip until P0.5 in mouse and migrate through the white matter to reach their mature sites by P10 (Englund et al., 2006).

**The external germinal layer (EGL)**
The external germinal layer (EGL) is the site of origin for granule cell precursors (GCPs) which come to be the most numerous neuron in the CNS. Formation of the EGL is dependent on Math1 (Ben-Arie et al., 1997). The EGL disperses from the rhombic lip at E17 and grows over the posterior cortical surface subpially, reaching the anterior pole at E20. It then extends back over the base of the cerebellum. Sonic hedgehog (SHH) secreted by Purkinje cells is a critical driver for GCP proliferation (Wechsler-Reya and Scott, 1999). The outermost EGL is the proliferating zone where GCPs undergo clonal expansion dividing every 18-20 h, after which cells undergo tangential migration to the inner differentiation zone. As GCPs differentiate
and extrude parallel fibres, their cell bodies are similarly oriented parallel to the longitudinal axis of the folium. The orthogonal orientation of GCPs in the outer and inner EGL is observable in transverse sections (Fig. 1.19). GCPs depart for the GL along Bergmann glia fibres approximately 20-48 h after they exit the cell cycle (Komuro et al., 2001). These migrate independently past Bergmann glia until the leading process reaches the white matter at the bottom of the GL (Jiang et al., 2008). The EGL is thickest at P10, after which it diminishes and disappears completely around P21.

**Figure 1.19.** Transverse section of P5 rat cerebellum. Granule cell precursors (GCPs) in the external germinal layer (egl) migrate from the upper proliferating zone (pz) to the lower differentiation zone (dz). Nuclei are elongated along the long axis of the folium as GCPs extrude parallel fibres as shown by hematoxylin nuclear stain. Afterwards the cell body departs through the molecular layer (ml) and PC layer (pcl) to the granule cell layer (gl). Nuclei detected by hematoxylin counterstain (blue). Source: M. Naughton. Scale bar = 50μm.

**Prospective white matter**

The prospective white matter emerges from an oval-shaped mass continuous with the cerebellar peduncles containing unmyelinated pre-cerebellar afferents and cerebellar efferents and traversed by migrating cells. This heterogeneous population includes fate-restricted precursors to interneurons, oligodendrocytes, astrocytes and microglia,
and also a subpopulation of multipotent neural stem cells (Lee et al., 2005a; Buffo and Rossi, 2013). There are no signs of lobulation until E21 in rodents, at which point the initial expansion of the hemispheres also occurs. Four lobular fissures (preculminate fissure, the primary fissure, the secondary fissure, the posterolateral fissure) advance and five trunks stem from the developing WM region. The anterobasal trunk develops into lobes I, II and III, the anterodorsal trunk forms IV and V, the central trunk forms VI, VII and VIII, the posterior trunk becomes IX and the inferior trunk develops into X (Altman and Bayer, 1996, p. 238). This is most advanced in the vermal region, with foliation and white matter partitioning being slower to proceed in the hemispheres. Myelination is not initiated until the end of the first postnatal week. At this time all lobules are well-developed and most neurons (except GCPs) have migrated and differentiated at their appropriate destinations.

1.4.11 Medulloblastoma

The germinal niches of the developing cerebellum give rise to the most common malignant paediatric brain tumours known as medulloblastomas. They fall into four molecular categories which each contain their own subgroups based on histopathology; WNT-activated, SHH-activated, Group 3 and Group 4 (Taylor et al., 2012; Louis et al., 2016). WNT medulloblastomas have the best prognosis with a long-term survival rate of over 90% (Taylor et al., 2012). Strong evidence suggests an etiological role of canonical WNT signalling in the occurrence of these tumours which are believed to stem from mossy fibre precursors. WNT tumours are generally located in the brainstem and around the 4th ventricle and may occur at any age. In contrast, most SHH tumours generally either occur between ages of 0-3 or over 16 years (Taylor et al., 2012). Approximately 25% of all medulloblastomas are of SHH subtype and stem from aberrant growth of GCPs in the cerebellar hemispheres (Gibson et al., 2010). This grouping is non-homogeneous however. It includes nodular-desmoplastic medulloblastoma which comprise 50% of SHH tumours. SHH/TP53 mutant tumours represent a separate, rare and high-risk group. Group 3 and Group 4 are less well-characterised. Group 3 has the poorest prognosis and is associated with MYC amplification whereas Group 4 has a better prognosis and is rarely associated with MYC or MYCN amplification.
1.4.12 Cerebellum pathology in Multiple sclerosis

The cerebellum is an important target in MS and is associated with persistent pathology and a poorer prognosis (Weier et al., 2015). The all-encompassing reach of the cerebellar connectome makes this region an eloquent area in MS that can give rise to a range of symptoms including the cerebellar motor syndrome and CCAS (Weier et al., 2015). The WM of the cerebellum is equally affected as in the forebrain in terms of plaque count per wet weight of tissue but cerebellar GM can be very heavily affected with some cases affecting up to 90% of the total area (Kutzelnigg et al., 2007; Gilmore et al., 2009; Howell et al., 2015; Weier et al., 2015).
1.5 RATIONALE

The oligodendrocyte is necessary for myelin production and maintenance in the CNS. It is critical for saltatory conduction, metabolic support and axonal health, all of which are damaged in demyelinating conditions such as MS. The requirement of OPCs to synthesise vast quantities of myelin membrane during terminal differentiation constitutes an increased load on the ER which may trigger the Unfolded Protein Response. Despite wide recognition of the substantial synthetic demands placed on myelinating oligodendrocytes, the potential role of the UPR has not been investigated to date. Assessing the activation of this pathway during in vivo developmental myelination would provide a basis for further study. The cerebellum is of clinical relevance to MS and is a widely-used model of myelination. The majority of these reports, however, do not take into account the well-established, topographical complexity of the cerebellum and the distinct molecular profiles of each domain. Several cerebellar disorders exhibit specific pathological patterns along these domains, several of which implicate UPR activation. It is not known whether UPR-associated molecules are differentially expressed in the cerebellum, therefore basal UPR expression should be assessed when employing this model.

1.6 HYPOTHESIS

The unfolded protein response is required for successful myelination by oligodendrocytes.

1.7 AIMS

- Profile physiological expression of UPR-associated proteins in normal cerebellum to screen for patterning.

- Conduct a comprehensive analysis of UPR signalling pathways during specific stages of myelination of cerebellar white matter tracts.

- Investigate if modulation of distinct UPR signalling pathways can positively or negatively regulate myelination in in vitro culture systems.
CHAPTER 2

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Equipment, instruments and plastic consumables

*CNS tissue dissection*

- Dissecting microscope
- Dissection board
- Guillotine
- No. 3 blade holder
- #10 and #11 scalpel blades (Swann-Morton)
- Bone scissors
- Iris scissors
- Rongeurs
- #5 forceps (Dumont)
- #7 forceps (Dumont)

*Histology*

- Leica ASP300 Automated Tissue Processor
- Leica EG1150 H Heated Paraffin Embedding Module
- Leica EG1130 Cold Plate
- Leica RM2235 Microtome
- Histology water bath
- Leica CM1850 Cryostat
- Cassettes
- Forceps
- Paintbrushes
- Superfrost Plus slides (Fisher Scientific)
- Tefal pressure cooker
- Stuart Infra-Red Hot plate CR300
- Filter paper (Fisher 11566873)
- Glass coverslips
**Imaging**

Olympus BX61 microscope
Olympus VS120 slide scanner

**Molecular Biology**

TissueLyser LT (Qiagen)
Stainless steel beads (Qiagen 69990)
Thermocycler
Applied Biosystems StepOne Plus Real-Time instrument
Rnase-free barrier tips
Rnase-free tubes

**Isolation of OPCs**

Rotary shaker
Filter paper (Fisher 11566873)

**Preparation of organotypic brain slice cultures**

McIlwain Tissue Chopper (GaLa Instrumente)
Double-edged blades
Silicone sheeting
Petri dishes
Cotton buds
Spatula
Pasteur pipettes
Scalpel blade No. 11 Swann-Morton (SCA-353-070V)
Millicell-CM 30mm well inserts (Millipore)

**Tissue culture**

Tissue culture incubator
Tissue culture water bath
Biosafety cabinet
Microscope
Pipette buoy
Serological pipettes (5, 10, 25 ml)
Pipettes (P2-P1000)
Pipette tips (10-1000μl)
0.5, 1.5 and 2.0 ml tubes

Other Tissue Culture plastics

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2.1.2 Reagents and chemicals

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52
**General chemicals**

- Sodium Hydroxide: Sigma-Aldrich 58045
- Hydrochloric acid: Sigma-Aldrich 320331
- Tris(hydroxymethyl)aminomethane: Sigma-Aldrich 25289
- Trizma base: Sigma-Aldrich T1503
- Ethylenediaminetetraacetic Acid: Fisher BP120-1

**Histology** (See Tables 2.1 and 2.2 for lists of antibodies)

- Paraformaldehyde: Sigma-Aldrich 158127
- Xylene: Fisher X/02200/17
- Ethanol: Lennox CRTSI0020716
- Industrial Methylated Spirits (IMS): Lennox CRTSI0330716
- Methanol: Fisher M/4056/17
- Acetone: Fisher 10194140
- 2-methylbutane (Isopentane): Fisher 167840010
- Hydrogen Peroxide 30%: Sigma-Aldrich H1009-100ml
- PBS tablets: Sigma-Aldrich P4417-100TAB
- Tween-20: Sigma-Aldrich P5927
- Triton-X 100: Sigma-Aldrich X100
- Luxol Fast Blue: Sigma-Aldrich S3382
- Gill’s No. 2 Haematoxylin: Sigma-Aldrich GHS216
- Eosin Y 1% (w/v) aqueous solution: Sigma-Aldrich E4009
- Acetic acid: Sigma-Aldrich 71251
- Lithium carbonate: Sigma-Aldrich 203629
- Normal goat serum: Thermofisher 31873
- Diaminobenzidine (DAB): Dako (Agilent) K3467
- VECTASTAIN Elite ABC HRP Kit: Vector Labs PK-6100
- VECTASTAIN ABC-AP Staining Kit: Vector Labs AK-5000
- Liquid Permanent Red: Dako (Agilent) K0640
Normal Goat Serum | Dako (Agilent) | X0907  
DPX mounting medium | Sigma-Aldrich | 6522  
OCT mounting medium | VWR | 411243  
Vectashield with DAPI | Vector Labs | H-1200  
Clear nail polish

**Molecular Biology**

**RNA Isolation**

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**DNase Treatment and cDNA synthesis**

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**Real-Time PCR**

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<td>Fast SYBR® Green Master Mix</td>
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### Tissue Culture Reagents

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<td>Sigma-Aldrich</td>
<td>D4263</td>
</tr>
<tr>
<td>Anti-O4, clone 81</td>
<td>Millipore</td>
<td>MAB345</td>
</tr>
<tr>
<td>AffiniPure Goat Anti-Mouse IgM</td>
<td>Jackson Immuno</td>
<td>115-005-020</td>
</tr>
<tr>
<td>PDGF</td>
<td>Peprotech</td>
<td>100-13A</td>
</tr>
<tr>
<td>bFGF</td>
<td>Peprotech</td>
<td>100-18B</td>
</tr>
<tr>
<td>Poly-D-lysine</td>
<td>Sigma-Aldrich</td>
<td>P6407</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>Sigma-Aldrich</td>
<td>P4707</td>
</tr>
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</table>
Mouse laminin Invitrogen 23017-015
T3 (3,3′,5-Triiodo-L-thyronine sodium salt) Sigma-Aldrich T6397
T4 (L-Thyroxine sodium salt pentahydrate) Sigma-Aldrich T2501
Tunicamycin Sigma-Aldrich T7765
Stealth RNAi™ siRNA Negative Control Life technologies 12935-300
Stealth siRNAs (Set of 3) RSS315363, RSS315364, RSS315365. Primer Set Atf6 Rat Life technologies 1330003
Lipofectamine® RNAiMAX Thermofisher 13778150
Guanabenz acetate Sigma-Aldrich G110
4μ8C Tocris 4479
L-α-Lysophosphatidylcholine (LPC) Sigma-Aldrich L4129-25MG
Horse Serum Sigma-Aldrich H1138
Glucose Sigma-Aldrich G7528

2.2 METHODS
2.2.1 ANIMAL HUSBANDRY
Sprague-Dawley rats were housed under standard conditions (12:12 h light:dark cycle with a room temperature of 21±2°C, humidity of 55±10% and water and food available ad libitum). Sprague-Dawley rats were bred in-house or obtained from Charles River Laboratories (Harlan). Prior to breeding males and females were housed in the same room to induce oestrus. Successful mating was indicated by presence of a vaginal plug. As this was difficult to observe consistently, the breeding female was placed into the cage of the male rat typically for up to two weeks or when positive signs of pregnancy were observed (abdominal swelling, enlargement of mammary glands and nipples). The male rat was removed prior to birth to avoid the risk of cannibalising young. Cages were monitored daily to record date of birth and number of pups per litter. Day of birth was designated as P0 (postnatal day 0). Animals were weaned at 21 days and placed in separate cages with same-sex littermates and used for breeding after reaching sexual maturity (3 months). Animals were sacrificed by decapitation until P14 and by CO₂ asphyxiation at older ages. All
experiments were approved by the NUI Galway Animal Care Research Ethics Committee (ACREC).

### 2.2.2 BRAIN DISSECTION

After sacrifice and decapitation, the head was dipped in ethanol and pinned to a dissection board. The skin was cut along the midline from the base of the skull to the eyes and cut again laterally from the base to remove the skin and expose the skull. In adult animals, rongeurs were used to remove bone overlying the cerebellum beginning from the foramen magnum, with particular care at the flocullonodular lobes. The whole brain could be exposed by then cutting the bone between the eye sockets and cutting along the sagittal suture with a large bone scissors until the frontal and parietal bones could be detached on either side. In younger animals, the calvaria could be removed in one piece without damage to the underlying tissue. Scissors were pointed at an outward angle, and the skull was cut laterally from the foramen magnum around the skull to the eye socket at either side. The bone between the eye sockets was cut and the skullcap carefully removed, using a curved forceps if required to separate the brain from the dura. A curved forceps was used to sever the olfactory bulbs from the forebrain. When tipped back, the brain tissue could come out easily, using a curved forceps to sever attaching cranial nerves to free the tissue. The brain was placed dorsal aspect upwards on a clean cutting surface. The hindbrain was removed from the forebrain by cutting between the superior and inferior colliculus with a No. 10 scalpel blade. The cerebellum was gently separated from the hindbrain using a No. 5 and No. 7 forceps by severing attachments from the cerebellar peduncles and any adjoining meningeal tissue. If removal of the meninges was required, the cerebellum was placed in a petri-dish with ice-cold dissection medium and gently teased apart under a microscope. Starting from its rostral surface, fine forceps were used taking care not to damage the cortical folds of the tissue. If the cerebella were to be sectioned sagittally, a clean single cut was made between the cerebellar hemispheres before fixation or embedding.
2.2.3 HISTOLOGY

2.2.3.1 Tissue fixation, paraffin-processing and embedding

For chromogenic immunohistochemistry, cerebella were quickly dissected and immersion-fixed in cold 4% (w/v) paraformaldehyde. Samples were laid in labelled cassettes and processed in a Leica ASP300 Automated Tissue Processor. The following program was selected:

1. 70% IMS for 1 hour
2. 90% IMS for 1 hour
3. 100% IMS for 1 hour
4. Xylene for 45 min
5. Xylene for 1 hour
6. Xylene for 1.15 hour
7. Paraffin wax at 60°C for 1 h
8. Paraffin wax at 60°C for 1 h
9. Paraffin wax at 60°C for 1 h

The tissue was embedded in molten paraffin wax and allowed to cool. The majority of sections were cut by Dr. J. McMahon as follows. Paraffin sections were cut on a Leica rotary microtome at 7 μm. Sections were floated onto a water bath at 55°C and lifted onto Superfrost Plus slides (Fisher Scientific). Sections were dried for 1 h at 60°C to remove wax and ensure good section adhesion. Slides were stored at room temperature in a dust-free environment.

2.2.3.2 General immunohistochemistry protocol

Blocking is performed with normal serum. Normal serum contains proteins and antibodies that will bind to non-specific epitopes in the sample, thus preventing conjugated antibodies from doing so. Commercial primary antibodies are raised from sera or cells of host species such as mouse and rabbit. The primary antibody binds to the antigen of interest, and the excess unbound antibody is washed away. The secondary antibody is directly or indirectly conjugated to a reporter. The secondary antibody is raised against the host species of the primary antibody. As the secondary antibody should only detect antibodies of a single species, dual-staining can be performed by selecting primary antibodies from different hosts with appropriate secondary antibodies utilising different reporters. The secondary antibody may be
directly conjugated to a reporter, such as a fluorophore and visualised by fluorescent microscopy. As the relationship between emitted light intensity and amount of fluorochrome is linear, if one fluorophore binds to a single epitope, it can be used to quantitatively assess expression of a protein. Alternatively, the secondary antibody may be indirectly conjugated to a reporter to allow amplification of signal. Conjugation with an amplified detection enzyme such as horseradish peroxidase (HRP), in the presence of a suitable substrate can induce a permanent reaction product that can be visualised by brightfield imaging. In the presence of hydrogen peroxide, the HRP enzyme causes oxidation of diaminobenzidine (DAB) chromogen and a permanent brown product is produced at the site of the target antigen. This is a more sensitive detection method as the ratio of reporter to each epitope is higher than for direct IHC and is useful for lowly-expressed proteins. Detailed IHC protocol variations are described below. Details of antibodies and conjugates used for immunohistochemistry are shown in Table 2.1.

2.2.3.3. Staining optimisation

Protocols were optimised for each individual antibody unless it had previously been performed by a member of the lab for the same product and manufacturer. Taking the suggested dilution from the respective datasheet and reported usage in the literature as a guide; low, high and mid-range dilutions of primary antibody (and secondary where applicable) were applied to sections with known expression of the antigen using an otherwise standard staining protocol (see below) (Fig. 2.1.). Optimisation was conducted alongside a negative antibody control and a positive staining control using an antibody with a known working dilution. If further alteration of antibody dilution failed to provide distinct signal:noise ratio, dilution or alteration of the blocking agent was varied for the blocking and/or antibody incubation steps. In frozen sections, optimal fixation was tested using methanol, acetone, 4% PFA-fixed and unfixed sections. Optimum dilutions for antibodies are shown in Table 2.1 and 2.2.
Figure 2.1. Example of staining optimisation of primary antibody concentration. Olig2 monoclonal antibody was applied to sagittal sections of P11 cerebellum at dilutions of 1 in 25, 50 or 100 in blocking solution alongside a section that underwent the same protocol without primary antibody.

Table 2.1. Primary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Name</th>
<th>Host/Isotype</th>
<th>Source</th>
<th>Cat No</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2B5</td>
<td>Mouse mAb</td>
<td>R&amp;D Systems</td>
<td>MAB1416</td>
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<td>Olig2</td>
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<td>MABN50</td>
<td>1:100</td>
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<td>Millipore</td>
<td>AB9610</td>
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<tr>
<td>MOG</td>
<td>Mouse mAb</td>
<td>Hybridoma supernatant, Prof. R. Reynolds</td>
<td>n/a</td>
<td>1:200</td>
</tr>
<tr>
<td>PLP/DM20</td>
<td>Mouse mAb</td>
<td>Millipore</td>
<td>MAB388</td>
<td>1:500</td>
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<tr>
<td>IBA1</td>
<td>Rabbit pAb</td>
<td>Wako</td>
<td>091-10741</td>
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<tr>
<td>GFAP</td>
<td>Rabbit pAb</td>
<td>Dako</td>
<td>Z0334</td>
<td>1:1000</td>
</tr>
<tr>
<td>ATF6</td>
<td>Rabbit pAb</td>
<td>Abcam</td>
<td>ab37149</td>
<td>1:100</td>
</tr>
<tr>
<td>phospho IRE1</td>
<td>Rabbit pAb</td>
<td>Novus</td>
<td>NB100-2323</td>
<td>1:100</td>
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<tr>
<td>alpha</td>
<td>Rabbit pAb</td>
<td>Novus</td>
<td>NB100-2323</td>
<td>1:100</td>
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<tr>
<td>phospho-PERK</td>
<td>Rabbit pAb</td>
<td>Santa Cruz</td>
<td>sc-32577</td>
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<td>BiP GRP78</td>
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<td>Abcam</td>
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<td>GRP94</td>
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<tr>
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Table 2.1. Primary antibodies used for immunohistochemistry (continued).

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<th>Cat No</th>
<th>Dilution</th>
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<tr>
<td>XBP1</td>
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<td>Mouse mAb</td>
<td>Sigma</td>
<td>G3893</td>
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<td>OX42</td>
<td>Mouse mAb</td>
<td>Millipore</td>
<td>CBL1512</td>
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<td>Mouse mAb</td>
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<td>MAB377</td>
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<td>Zebrin II</td>
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<td>Hybridoma supernatant, Prof. R. Hawkes.</td>
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<td></td>
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<td>AB1778</td>
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<td>MBP</td>
<td>Mouse mAb</td>
<td>Millipore</td>
<td>#05-675</td>
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Table 2.2 Secondary antibodies used for immunohistochemistry.

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<th>Dilution</th>
</tr>
</thead>
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<tr>
<td>Biotinylated Anti-Rabbit Ig</td>
<td>Swine pAb</td>
<td>Dako</td>
<td>E0353</td>
<td>1:400</td>
</tr>
<tr>
<td>Biotinylated Anti-Mouse Ig</td>
<td>Rabbit pAb</td>
<td>Dako</td>
<td>E0354</td>
<td>1:400</td>
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<tr>
<td>Universal ImmPRESS HRP</td>
<td>n/a</td>
<td>Vector</td>
<td>MP-7500</td>
<td>Neat</td>
</tr>
<tr>
<td>EnVision+ HRP (for mouse Ab)</td>
<td>n/a</td>
<td>Dako</td>
<td>K4010</td>
<td>Neat</td>
</tr>
<tr>
<td>EnVision+ HRP (for rabbit Ab)</td>
<td>n/a</td>
<td>Dako</td>
<td>K4006</td>
<td>Neat</td>
</tr>
<tr>
<td>Cy3 conjugated streptavidin</td>
<td>n/a</td>
<td>Jackson Immuno Research</td>
<td>016-160-084</td>
<td>1:4000</td>
</tr>
<tr>
<td>Alexafluor 488 (anti-mouse)</td>
<td>Goat pAb</td>
<td>Invitrogen</td>
<td>A21042</td>
<td>1:1000</td>
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</table>

2.2.3.4 Luxol Fast Blue/ Hematoxylin & Eosin (LFB/H&E)

All paraffin sections were stained with LFB/H&E to allow general assessment of tissue anatomy. Luxol fast blue is a histological stain used to stain myelin a blue-green colour in FFPE CNS tissue or frozen sections. Gill’s No. 2 haematoxylin is a progressive nuclear stain that stains nucleic acid a blue-purple colour. Eosin Y is a pink dye that stains cytoplasmic proteins.
Sections were dewaxed in two changes of xylene for 5 minutes each. Slides were immersed in 100% IMS, 100% IMS, 95% IMS for one minute each, followed by immersion in LFB solution (0.1% LFB, 0.5% acetic acid, 95% ethanol) overnight at 37°C. Slides were placed in 100% IMS to remove excess LFB and then differentiated by serial immersion in a) lithium carbonate, b) 100% IMS c) distilled H₂O to stop the reaction. This series was repeated until satisfactory distinction of myelinated axons between WM and GM layers was apparent under a microscope. Slides were immersed in Gill’s haematoxylin for ~30s until the requisite blue colour was achieved and then placed under running tap water (incubation time with hematoxylin could vary depending on how long the bottle had been opened and the level of oxidation that occurred). Sections were monitored under a microscope and counterstained with Eosin Y for 20-30 s. Sections were finally dehydrated in two changes of 100% IMS for 30 s and cleared with two changes of xylene for 5 min each. Slides were mounted and cover slipped with DPX.

2.2.3.5 Chromogenic immunohistochemistry

Unless otherwise stated, all chromogenic immunohistochemistry was carried out using Envision HRP secondary antibodies in a standard three step protocol with diaminobenzidine (DAB). Negative “no primary antibody” controls were included for all staining procedures.

2.2.3.6 De-waxing, endogenous peroxidase block, heat-induced antigen retrieval

Paraffin-embedded tissue section slides were placed in two changes of xylene for 5 min each. Slides were rehydrated in a series of graded IMS solutions (100%, 90%, 80%, 70%, 60%, 50%) for 30s-1min each followed by running tap water for 5min. Endogenous peroxidases were blocked using a 1 in 10 dilution of 30% H₂O₂ in 100% IMS or methanol for 20 minutes. Slides were then washed in water for 5min. Unless otherwise stated, heat-induced antigen retrieval was performed in a pressure cooker with 2L of 1X Tris-EDTA pH9 solution. Slides were placed inside within a slide holder, the lid was locked and the pressure cooker was heated by an infra-red hotplate at maximum power. The incubation was timed for 3 minutes once the visual
indicator of the pressure cooker showed it was at pressure. The pressure cooker was then placed into a large bath of cold water. Cold water was poured over the pressure cooker to reduce heat as quickly as possible. When the visual indicator showed it was no longer at pressure, the cooker was opened and the slide holder was rapidly transferred into water with a forceps to prevent drying out. Slides were allowed to cool before proceeding with staining protocol.

2.2.3.7 Standard chromogenic three step staining protocol
Sections were blocked with 5% normal goat serum (NGS) in PBS for 30 minutes at room temperature (RT). Primary antibody dilutions were made in blocking solution and incubated overnight at 4°C. Slides were washed for 5 min three times in PBS. Unless otherwise indicated, sections were incubated with appropriate Envision HRP secondary antibody neat for 30 min at room temperature. Slides were washed three times in PBS. A fresh aliquot of diaminobenzidine (DAB) solution was thawed and filtered for each run. It was activated with 1μl of 30% hydrogen peroxide per ml of DAB solution immediately prior to use and thoroughly mixed. The activated solution was applied to all sections and development of the chromogen was monitored microscopically. DAB was incubated for a maximum of 3 minutes or stopped when background staining appeared to increase. The reaction was stopped with rapid application of H2O to all slides and immersion for 5 min. Sections were incubated with Gill’s No. 2 haematoxylin counterstain until required blue colour was achieved and washed thoroughly with tap water. Sections were dehydrated with three changes of 100% IMS for 1 min each. Sections were cleared in two changes of xylene for 5 min each and mounted onto Superfrost slides with DPX mounting medium.

2.2.3.8 Streptavidin biotin ABC-HRP method
Biotin-conjugated (biotinylated) secondary antibodies are used for amplification of signal. Avidin has very strong affinity for biotin for which it has four separate binding sites. When avidin (contained in reagent A) and a biotinylated HRP enzyme (contained in reagent B) mixture is incubated on a section, a macromolecular complex (ABC) can form at the site of the biotinylated secondary, regardless of what host the antibody was raised in. This complex amplifies the signal detected by DAB at the site of antigen expression in the tissue.
After primary antibody incubation, slides were washed three times in PBS. Sections were incubated with appropriate biotinylated secondary antibodies diluted 1:400 in PBS for 30 min at RT. Meanwhile, the ABC horseradish peroxidase-labelled complex was prepared using Vectastain Elite ABC HRP reagent. Two drops of reagent A were added to 5 ml of buffer, followed by two drops of reagent B to the same tube and mixed thoroughly. The solution was allowed to stand for 30 minutes before use. Sections were washed three times in PBS before incubation of the ABC solution for 30 min at RT. Sections were washed three times in PBS before proceeding with chromogen development with DAB.

2.2.3.9 Universal ImmPRESS HRP method
This method employs a polymer-conjugated HRP detection reagent designed for use with both rabbit and mouse primary antibodies for amplification of signal.

After primary antibody incubation, slides were washed three times in PBS. Sections were incubated with universal ImmPRESS reagent for 30 min at RT. Sections were washed three times in PBS before proceeding with DAB chromogen development.

2.2.3.10 Alkaline phosphatase / Liquid Permanent (AP/LPR) method
This detection system employs an alternative reporter enzyme alkaline phosphatase (AP). The AP-conjugated streptavidin binds strongly to the biotinylated secondary antibody at the target site of the antigen. Detection is visualised by Liquid Permanent Red (LPR) which is a substrate that forms a permanent red reaction product on reaction with AP.

After primary antibody incubation, slides were washed three times in PBS. Sections were incubated with appropriate biotinylated secondary antibodies diluted 1:400 in PBS for 30 min at RT. Slides were washed three times in PBS and incubated with streptavidin alkaline phosphatase for 30 min at RT. Slides were washed in PBS followed by TBS for 5 minutes each. Liquid permanent red (LPR) chromogen was allowed to equilibrate at RT for 20 minutes before use. 3 ml of chromogen buffer and 1 drop of LPR were added together, mixed and incubated on sections for up to max. 20 minutes at RT. Slides were monitored microscopically during development.
of chromogen and the reaction was stopped with H₂O. Slides were allowed to air dry fully before mounting onto coverslips with DPX.

2.2.3.11 Immunostaining Procedure for Dual Colometric staining
Paraffin embedded sections were stained using the standard three step DAB protocol as described. After development of the chromogen DAB, slides were washed in water for 5 minutes. Sections were blocked for endogenous peroxidases with a 1 in 10 dilution of hydrogen peroxide solution in IMS for 10 min at RT, and then washed for 5 min in tap water. Sections were blocked with 5% NGS for 30 min and incubated with a primary antibody of a different host in blocking solution overnight at 4°C. The protocol for AP/LPR detection was then followed as described above.

2.2.3.12 Cryo-embedding and cryo-sectioning
For fluorescent staining, freshly harvested tissue was embedded in OCT (VWR International Ltd, Dublin, Ireland) on a cork base immediately after dissection and snap-frozen in isopentane cooled in a liquid nitrogen bath. Tissue blocks were stored at -80°C. Ten μm frozen sections were thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Dublin, Ireland) and stored at -80°C until use.

2.2.3.13 Immunofluorescent dual-labelling
Frozen sections were brought to room temperature and air-dried prior to incubation in ice-cold methanol for 20 minutes. All sections were blocked in 5% normal goat serum (NGS) in PBS for 30 minutes and incubated overnight with primary antibody diluted in 5% NGS at 4°C. Sections were washed twice in PBS for 5 min. The signal for rabbit polyclonal primary antibodies was amplified by incubating sections in a biotinylated swine anti-rabbit secondary antibody dilution (1:400 in PBS) for 30 minutes. Sections were washed twice in PBS for 5 min before application of streptavidin-Cy3 and goat anti-mouse Alexafluor 488 (1:1000 each in PBS) for 30 min at room temperature (Table 2.1). All slides were mounted in Vectashield containing diamino-2-phenylindole (DAPI) (Vector) to allow visualisation of nuclei.
Coverslips were sealed with nail varnish. Negative “no primary antibody” controls were included for each staining run (Appendix Fig. 2.1).

2.2.3.14 Wholemount immunohistochemistry

Rat cerebella from were stained for known striping markers and UPR-associated molecules using a published protocol (Sillitoe and Hawkes, 2002). All steps were performed with gentle rocking and at 4°C unless specified otherwise. Cerebella were first placed in Dent’s fixative (4:1 methanol (MeOH): dimethyl sulfoxide (DMSO)) overnight. They were then immersed in Dent’s bleach (4:1:1 MeOH:DMSO:30%H2O2) for 8 h or until tissue was completely white. The cerebella were placed in two washes of 100% MeOH for 30 min before undergoing 4-5 cycles of freeze-thaw from -80°C to RT. At this point it could be stored in 100% MeOH at -80°C until needed.

The tissue was rehydrated in 50% methanol, 15% methanol, and PBS for 90 min each at RT. Enzymatic digestion was carried out with 10 μg/ml proteinase K (600U/ml) in PBS for 5 min at RT to facilitate reagent penetration. Cerebella were then rinsed three times in PBS for 10 min each. Non-specific antibody binding was blocked with 2% non-fat skim milk powder, 0.1% Triton X-100 in PBS (PBSMT) for 6-8 h at RT, changing PBSMT every 2-3 h. The tissue was incubated in primary antibody diluted in PBSMT with 5% (v/v) DMSO for 48 h at 4°C. The tissue was rinsed three times with PBSMT for 2-3 h each at 4°C and then incubated overnight with secondary antibody in PBSMT and 5% (v/v) DMSO at 4°C. This was followed by three to four changes of PBSMT for 2 to 3 h each at 4°C, followed by a final overnight incubation at 4°C. A final wash with 0.2% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100 in PBS for 2 h at RT was carried out before incubation with freshly prepared DAB solution activated with H2O2. Once desired colour intensity was reached the reaction was stopped with 0.04% sodium azide in PBS. Images were captured on X microscope while the cerebella were immersed in 0.1% Triton-X 100 in PBS.
2.2.4 IMAGE QUANTIFICATION

2.2.4.1 Quantification of positive cells in cerebellar white matter tracts.
Fields in cerebellar lobules III and IV (Fig. 2.2) of FFPE DAB stained sections at P7, P10, P14, P17 and adult (n=3-6 animals) were used for quantification of positive cells of UPR markers. Images from all timepoints of a respective antibody stain were captured at the same magnification either on an Olympus BX61 or captured with OlyVIA software from whole slides scanned at 40X magnification on an Olympus VS120 slide scanner and saved as .tif files. Scales were adjusted as necessary for accurate area quantification. Random.org was used to create a string of random file IDs for blinding. This service generates true random numbers and strings based on atmospheric noise (Fig. 2.3). The original image files were listed in alphabetical order in Microsoft Excel and assigned new IDs according to the randomly generated list. The files were renamed using Bulk Rename Utility software and were automatically randomised when placed in the new alphabetical order (Table 2.3). This ensured that the assessor was blinded before analysis and that the effect of any subjective change in cell counting analysis over time would be attenuated by randomisation.

Figure 2.2. Fields used for quantification of white matter tracts. Fields were taken from cerebellar lobes III and IV only, at all time points
Figure 2.3. A sample of random strings generated by random.org. Strings were used for renaming files.

Table 2.3. Simultaneous randomisation and blinding of image files.

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<th>Old order</th>
<th>Original File Name</th>
<th>New File ID</th>
</tr>
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<td>S7VWG</td>
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<tr>
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<td>AUF 1301 PDI A 41F</td>
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<td>PO0AU</td>
</tr>
<tr>
<td>13</td>
<td>AUF 1310 PDI B 31F</td>
<td>TMG3S</td>
</tr>
<tr>
<td>14</td>
<td>AUF 1310 PDI B 41F</td>
<td>VF4GG</td>
</tr>
<tr>
<td>15</td>
<td>AUF 1311 PDI 31F</td>
<td>7 YYV0</td>
</tr>
<tr>
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<td>AUF 1311 PDI 41F</td>
<td>1VIDB</td>
</tr>
<tr>
<td>17</td>
<td>AUF 1314 PDI A 31F</td>
<td>V1OTA</td>
</tr>
<tr>
<td>18</td>
<td>AUF 1314 PDI A 41F</td>
<td>BASE9</td>
</tr>
<tr>
<td>19</td>
<td>AUF 1317 PDI B 31F</td>
<td>572OT</td>
</tr>
<tr>
<td>20</td>
<td>AUF 1317 PDI B 41F</td>
<td>7ZC8C</td>
</tr>
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</table>

<table>
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<th>New File ID</th>
<th>Old order</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>2</td>
<td>1VIDB</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>20JMA</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>2Q6AQ</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>56GTX</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>572OT</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>5DMBH</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>5NRU9</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>7YYV0</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>7ZC8C</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>BASE9</td>
<td>18</td>
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<td>11</td>
</tr>
<tr>
<td>13</td>
<td>PO0AU</td>
<td>12</td>
</tr>
<tr>
<td>14</td>
<td>S7VWG</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>SC483</td>
<td>10</td>
</tr>
<tr>
<td>16</td>
<td>TMG3S</td>
<td>13</td>
</tr>
<tr>
<td>17</td>
<td>V1OTA</td>
<td>17</td>
</tr>
<tr>
<td>18</td>
<td>VF1BW</td>
<td>3</td>
</tr>
<tr>
<td>19</td>
<td>VF4GG</td>
<td>14</td>
</tr>
<tr>
<td>20</td>
<td>ZLH3S</td>
<td>4</td>
</tr>
</tbody>
</table>

A sample list of image files that were assigned new file IDs from randomly generated strings using random.org. Renaming was automated using Bulk Rename Utility software and the files were automatically randomised in their new alphabetical order.
White matter tracts (outlined by the internal granular layer) were manually defined and cropped in Fiji ImageJ (Fig. 2.4). This step was found to aid blinding as cues in the cortical layers could inform the approximate age of the animal (e.g. relative depth of EGL/IGL, Purkinje cell morphology, etc.). In addition, staining in the cortical layers could change dramatically with age including changes in background glomerular staining in the IGL. This detracted from more subtle staining patterns occurring in the WM and could affect manual counts between groups. The blinding step was then refined so that it was applied after images were already cropped to display only the WM (Fig. 2.4, top right). These renamed and randomised files were used for blinded, manual counting of positive cells and the data input into Microsoft Excel. Marking of positive cells ensured that cells were not accidentally missed or double-counted (Fig. 2.4, bottom left).

**Figure 2.4.** Image processing for quantification of area and positive cells in cerebellar WM tracts. The original file (top left) was cropped to reveal only the WM by manually outlining the borders of the internal GL and saved as a new file (top right). This was used for manual counting of positive cells (bottom left) and for automated measurement of WM area in ImageJ. The mask of the quantified area (bottom right) was used for quality control.
The cropped file was also used for automated measurement of the WM area in ImageJ. A macro was used to automate this process for all files of the same cohort as shown below.

```java
run("8-bit");
setAutoThreshold("Default");
// run("Threshold...");
setThreshold(0, 249);
run("Convert to Mask");
run("Set Scale...", "distance=3700 known=1 pixel=1 unit=mm global");
run("Analyze Particles...", "size=0-Infinity circularity=0.00-1.00 show=Masks display clear summarize");
run("Measure");
```

The mask of the quantified area (Fig. 2.4, bottom right) was used as a quality control check. The total cell number and mean cell density per time point were assessed by nuclear staining. Counts of positive cells were expressed as a percentage of total cell number to account for changes in cell density over time. Data were only unblinded after all files from all timepoints for the respective antibody stain had been quantified and were ready for statistical analysis.

### 2.2.4.2 Quantification of glia in cerebellar white matter tracts

Oligodendrocytes were identified by expression of the cell lineage transcription factor olig2. This nuclear stain allowed simple quantification of positive cells as detailed above. No specific nuclear marker was available for either astrocytes or microglia. Astrocytes and microglia were identified by expression of GFAP and Iba1 markers which stain the cell bodies and processes of the respective cell types. As both cell types mature into highly ramified cells in white matter tracts, it was not possible to clearly distinguish single cells. Therefore, in lieu of counts of positive cells, the respective percentage area stained by either GFAP or Iba1 was quantified for each timepoint. Threshold parameters were optimised in Fiji ImageJ for detection of DAB product on the basis of colour separation using test images from different lobules of the same cohort. Using the HSB colour space of the Threshold Colour function, Hue was set at 130-230 with ‘Pass’ unticked; Saturation was set at 51-255 with ‘Pass’ ticked, Brightness set at 0-255, ‘Pass’ ticked. This allowed for specific detection of DAB product which could then be quantified (Fig 2.5).
Figure 2.5. Colour thresholding of a GFAP DAB stained image counterstained with hematoxylin. Optimised HSB thresholding parameters in Fiji ImageJ allowed specific detection of DAB product on the basis of colour separation.

Once parameters were optimised, the selected area of DAB staining could be converted to a binary mask to allow area measurements using the following macro.

```java
run("8-bit");
setAutoThreshold("Default");
//run("Threshold...");
setThreshold(0, 0);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Make Binary");
run("Convert to Mask");
run("Measure");
```

The same protocol was applied to all blinded images and automated using the Fiji>Process>Batch>Macro function. The mask files created were used for quality control to ensure the area measured was specific to the staining observed (Fig. 2.6). The area of staining was expressed as percentage of the total area of the respective WM tract and analysed using GraphPad Prism.
Figure 2.6. Quantification of GFAP staining area using colour thresholding. Image processing was automated through Fiji ImageJ Batch>Macro function after optimisation of threshold parameters. Thresholding was applied to the image of the outlined WM tract (top left) which enabled specific detection of GFAP (top right). A new image of the binary mask was created and used for quantification of staining area (bottom left). This analysis produced a mask of the exact area which had been quantified that could be rechecked for quality control purposes (bottom right).

2.2.4.3 Quantification of brightfield images in OPC cultures

Images were captured at 20x magnification from a tissue culture brightfield microscope from a minimum of 3 fields each from 4 wells per condition. Cells were counted and scored manually for morphological features of maturation according to the following scale: I) bipolar/tripolar cells with simple processes, II) cells with increased secondary processes III) cells with increased primary and secondary processes, IV) highly complex cells with extensive tertiary processes, V) terminally differentiated cells with evidence of membrane expansions outside the cell body (Fig. 2.7).
2.2.5 MOLECULAR BIOLOGY METHODS

2.2.5.1 RNA extraction

RNase-free tubes were labelled and prepared in advance in the fume hood with 0.5ml Trizol/TriReagent per tube per 5x10⁶ cells, 50mg of tissue or per three organotypic slices. If homogenising tissue or slices immediately, a 7mm RNase-free sterile stainless-steel bead was placed into each sample tube.

Cells were washed in PBS before resuspending directly in Trizol/TriReagent and pipetted up and down 20 times. For tissue, the tissue of interest was dissected immediately after sacrifice and placed directly into the labelled tube. For organotypic slices, the membrane inserts were dipped in ice-cold PBS. The slices were transferred from each well to the appropriate tube by suction using the pipette. Samples could be stored at -80°C until use.

Homogenisation of tissue or slices: Sample tubes containing steel ball and tri-reagent/trizol were placed into the adapter for the TissueLyser so that it was balanced on each side. The knob of the lid was screwed tightly until secured and operated for 1 minute at 30Hz. The tubes were placed immediately on ice and centrifuged briefly at 4°C. The lysate was transferred to a fresh RNase-free tube. The steel balls had to be decontaminated, washed and sterilised before re-use.

The samples were allowed to stand for 5 minutes at room temperature to promote dissociation of nucleoprotein complexes. 100 μl of chloroform was added to each tube (0.2ml chloroform per 1ml Trizol/TriReagent), shaken vigorously for 15 seconds and allowed to stand for 5 minutes at room temperature. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube. (After centrifugation, the sample separates into 3 phases. The upper, colorless, aqueous phase contains RNA; a white interphase; and a
lower, red, organic phase. Tissues with an especially high fat content, may have an additional clear phase below the red, organic phase. The volume of the aqueous phase is approx. 60% of the volume of the Trizol/TriReagent lysis reagent originally added.)

[Optional step to aid visualisation of RNA pellets (recommended for organotypic slices and very small samples). 1µl of GlycoBlue and 3M sodium acetate was added to the aq. sample in a 1:5 ratio (e.g. 40µl sodium acetate to 200µl of sample for a final 0.5M concentration).]

250 µl of isopropanol was added to the sample (500 µl of isopropanol per 1ml of Trizol/TriReagent) and thoroughly mixed by vortexing. The tubes were placed in -20°C. (The sample can be placed at -20°C overnight or continue to the next step after 15 min. The interphase and organic phase can be stored overnight at 4°C for DNA and protein extraction.)

The sample was allowed to stand for 10 minutes at room temperature and then centrifuged at 12,000 g for 10 minutes at 4°C. The RNA will form a pellet on the side and bottom of the tube and should be blue if GlycoBlue is used. The supernatant was removed and the RNA pellet was washed by adding 0.5 ml of 75% ethanol (1 ml of 75% ethanol per 1ml Trizol/TriReagent). The sample was centrifuged at 7,500 g for 5 minutes at 4°C. The ethanol wash step and centrifugation was repeated one more time. If the pellet floats, repeat centrifugation.

The supernatant was completely removed and the pellet allowed to air-dry for 5-10 minutes. 20 µl or appropriate volume of RNase-free water was added and mixed by repeat pipetting. (If pellet is difficult to dissolve mix with a micropipette at 55-60°C for 10-15 minutes). The sample was placed on ice as soon as possible, aliquoted to appropriate volumes and quantified by NanoDrop in ‘RNA mode’ and stored at -80°C.

2.2.5.2 DNase digestion

DNase digestion was conducted according to manufacturer’s instructions (Cat. No. 18068-015 Invitrogen). To an Rnase-free 0.5ml tube on ice, 500ng RNA, 1 µl of 10X rxn buffer,1 µl of Dnase I and appropriate volume of RNase-free H2O was added for a final volume of 10µl per tube. An RNA negative control was included
for which H₂O was used as a substitute. An RT negative control sample was also included for which RNA was taken from any other sample. Tubes were incubated for 15 min at RT. Dnase I was inactivated by addition of 1µl 25mM EDTA. The tubes were placed in a thermocycler and heated for 10 min at 65°C, then chilled at 4°C (program MN1). The samples were then ready for reverse transcription (final volume 11µl per tube).

2.2.5.3 cDNA synthesis using SuperScript II Reverse Transcriptase
Each sample tube contained 11µl of 500ng RNA after DNase digestion. 10mM dNTP mix was prepared in advance from dATP, dCTP, dGTP, dTTP stocks in RNase-free H₂O (Promega). Using random primers and SuperScript II Reverse Transcriptase kit (Invitrogen), two mixes were prepared according to the number of sample tubes as shown in Table 2.4.

Table 2.4. cDNA synthesis reagent preparation.

<table>
<thead>
<tr>
<th></th>
<th>(µl) per tube</th>
<th>No. tubes</th>
<th>Reqd. Vol.</th>
<th>Reqd vol+10%</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Random primers</td>
<td>1 tube</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>Tube Mix 1</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1 tube</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>5X buffer</td>
<td>4 tubes</td>
<td>40</td>
<td>44</td>
<td></td>
<td>Tube Mix 2</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2 tubes</td>
<td>20</td>
<td>22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To each tube, 2µl of Tube Mix 1 was added. The samples were incubated in a thermocycler at 65°C for 5 minutes to denature target and enzymes and chilled at 4°C for 5 min (MN2). The tubes were briefly centrifuged before addition of 6µl of Tube Mix 2. The tubes were vortexed gently and allowed to incubate for 2 min at RT. 1 µl of SuperScript II RT enzyme was added to all except the RT negative control (replaced with RNase-free-H₂O) and mixed by pipetting gently up and down. The tubes were incubated at RT for 10 minutes, vortexed gently and placed in the thermocycler using the following programme: Annealing 25°C for 10 min, Extension 42°C for 50min, Heat-inactivation of RT enzyme 70°C for 15 min, 4°C until end. Following this, 180µl of H₂O was added in order to dilute the cDNA 1 in 10 in preparation for qPCR. Samples were aliquoted and stored at -20°C until use.
2.2.5.4 \textit{qPCR}

Based on a total 20µl reaction volume in a 96 well plate, a primer mix was prepared for each gene of interest depending on the number of reactions as shown in Table 2.5.

\textbf{Table 2.5. Preparation of master mixes for qPCR.}

<table>
<thead>
<tr>
<th>Master Mix</th>
<th>Vol for 1 rxn (µl)</th>
<th>Vol reqd+10% (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast Sybr Green Master Mix</td>
<td>10</td>
<td>352</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2</td>
<td>70.4</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2</td>
<td>70.4</td>
</tr>
<tr>
<td>Water</td>
<td>4</td>
<td>140.8</td>
</tr>
<tr>
<td>cDNA Template</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

\textbf{No. of rxns} 32

An example of a Sybr Green mastermix preparation template for genes of interest based on the total number of 32 reactions and on a 20µl total volume per well.

For each gene of interest, 18µl of the relevant mastermix was added to appropriate wells in a 96 well reaction plate, after which 2µl of relevant cDNA template was added. The plate was sealed, wrapped in foil and centrifuged so that all reaction components were at the bottom of the well. The plate could be stored at 4°C in the dark for up to 48 h until the qPCR machine became available. Using an Applied Biosystems StepOne Plus Real-Time PCR instrument, the following program was used for Fast Sybr Green relative quantitation experiments (Table 2.6).

\textbf{Table 2.6. StepOne Plus program used for Fast Sybr Green qPCR}

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Duration</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Fast DNA Polymerase, UP Activation</td>
<td>95°C</td>
<td>20s</td>
<td>HOLD</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>3s</td>
<td>40</td>
</tr>
<tr>
<td>Anneal/Extend</td>
<td>60°C</td>
<td>30s</td>
<td></td>
</tr>
</tbody>
</table>

This was followed by melt curve analysis to assess the specificity of primer product. All primer concentrations were optimised and annealing temperature was varied.
where necessary. Standard curves to determine the efficiency of each primer set were performed using serial dilutions of cDNA (see appendix Fig. A2.1).

### 2.2.6 OPC PURIFICATION BY IMMUNOPANNING

Immunopanning methods were adapted from protocols provided by Prof. R. Reynolds (Imperial College London, personal communication) and those published in the literature (Dugas and Emery, 2013). The following reagents and materials were prepared in advance. The volumes indicate the amount required per immunopanning plate. One immunopanning plate is sufficient for cell isolation from four P4 rat pups, or two P6-8 rat pups. Note that PBS without Ca\(^{2+}\)/Mg\(^{2+}\) was only used during tissue dissection, normal PBS should be used for all other steps.

#### 2.2.6.1 Preparation

- 50 mM Tris-HCl (pH 9.5).
- 2% BSA in HBSS (pH 7.4).
- 10cm Petri dishes coated with 30 µl of goat anti-mouse IgM (2.3-2.4mg/ml) in 10 ml of sterile 50 mM Tris-HCl (pH 9.5) and allowed to incubate overnight at 4°C.
- Dissection medium (at least 20ml): DMEM (D6429), 1% P/S, 1% L-G.
- Pre-plating medium for adherent cells (20ml per immunopanning plate plus 40ml): DMEM (D6429) 1% P/S, 1% L-G, 5% FBS.
- 8ml per immunopanning plate: DMEM (D6429), 1% P/S, 1% L-G, 4% BSA.
- Panning buffer/Suspension medium (10ml per plate): DMEM (D6406), 1% P/S, 1% L-G, 5% FBS, 1% N2.
- 5 ml of 0.2% BSA solution in HBSS per plate.
- 10ml of 1X SD mix per plate (3mg/ml BSA, 0.5mg/ml SBTI, 80U/ml DNase)
- 6ml of BSA/SBTI stock per plate (6mg/ml BSA, 1mg/ml SBTI)
- 10 ml per plate of filtered 30% FBS in HBSS

Dissecting instruments were sterilised in advance by autoclaving. 10ml of papain and DNase solution was prepared fresh on the day of isolation. Two vials of papain (≥100U per vial) were reconstituted in 8 ml HBSS and placed in a waterbath to dissolve. A vial of DNase (2000U) was reconstituted in 2 ml HBSS and added to the
papain to get a final solution of 20U/ml papain and 200U/ml DNase which was sterile-filtered. Immunopanning plates were washed three times with HBSS and incubated with 5μg (5-10μl, depending on the lot no.) of O4 antibody in 5ml of 0.2% BSA and incubated for over 2 h at RT.

A dissection board was prepared in a biosafety cabinet with sterile drapes. Filter paper was soaked with 70% ethanol and left to dry in the sterile area. The animals were sacrificed by decapitation. The head was dipped in 70% IMS and placed on the dissection board in the hood. The brain was dissected as previously described. The cortex was isolated by removal of the cerebellum, brainstem, midbrain and hippocampi. The meninges were removed by rolling the outer cortical tissue on the filter paper. The tissue was placed in ice-cold dissection medium in a 10 cm petri-dish until cortical tissue from all animals were pooled together.

2.2.6.2 Digestion

Excess liquid was removed from the petri-dish containing the cortical tissue. The cortical tissue was diced for ~5 minutes with a No. 10 scalpel blade, occasionally turning the petri-dish at 90 degree angles during chopping until it was in ~1 mm$^3$ chunks. The tissue was transferred to a standard T25 flask (2 brains per flask). All tissue pieces were collected with washings using the papain/DNase solution. The remainder of papain/DNase solution was added to the T25 flask and placed in an incubator at 37˚C for 90 min, with gentle agitation every 15 min by gently rocking the flask.

The tissue was transferred to a 50ml tube and allowed to settle. As much overlying papain solution was removed as possible. Any remaining tissue pieces in the digestion flask were removed with 2 mL of SD mix and added to the 50 ml tube and mixed to stop papain digestion. The tissue was allowed to settle once again. Overlying SD mix was removed from the tissue and a fresh 2 mL of SD mix added.

2.2.6.3 Dissociation

Dissociation was performed in SD mix because the cells survive trituration better in a lower protein solution. BSA/SBTI stock was subsequently used to fully quench any residual enzymatic activity. The tissue was mixed through a 5 ml serological pipette and allowed to settle for 1-2 min. The overlying cell suspension was transferred to a fresh tube. One ml of SD mix was added and the dissociation repeated through the
5ml serological pipette. After settling for 1-2 min the overlying cell suspension was again added to the tube. This process was repeated with 1ml of SD mix triturated through a P1000 sterile barrier tip until all of the tissue was dissociated and all the prepared SD solution was used up.

The cells were centrifuged at 1200rpm for 15 min at RT. The supernatant was discarded, being careful not to disturb the cell pellet. The pellet was resuspended in BSA/SBTI stock solution. This suspension was centrifuged at 1200rpm for 15 min at RT. The supernatant was discarded and the pellet resuspended in 10ml of pre-plating medium per immunopanning plate. This suspension was plated into standard T75 flask and incubated at 37ºC for 25 min with gentle agitation mid-way through to allow microglia, astrocytes and other cells to adhere. Cells remaining in suspension were removed and spun at 1200 rpm for 5 min at RT.

2.2.6.4 Immunopanning

The cell pellet was resuspended in 10 ml of panning buffer/suspension medium per plate (DMEM D6406, 5% FBS, + N2). The O4 antibody was washed off the panning plate with HBSS three times, then washed with remaining 10 ml of pre-plating medium. The cell suspension is distributed with 10 mls of cells into each panning plate and incubated for 45 min at RT, agitating every 15 min so that all cells have an opportunity to adhere. During incubation, 10x trypsin stock was thawed and a bottle each of HBSS with Ca²⁺/Mg²⁺ and without Ca²⁺/Mg²⁺ (modified) were allowed to equilibrate at RT. The immunopanning plates were shaken and the non-adherant cell suspension was poured off. The plates were shaken and rinsed thoroughly with HBSS with Ca²⁺/Mg²⁺. The removal of non-adherent cells was checked under the microscope.

2.2.6.5 Trypsinisation

The O4 immunopanning plate was rinsed in HBSS without Ca²⁺/Mg²⁺ and then incubated in 4ml of trypsin solution. OPCs adhere very strongly to the O4 antibody and the standard 1x trypsin concentration took over 15 min at 37°C. 4ml of 2x trypsin in HBSS without Ca²⁺/Mg²⁺ was used in subsequent immunopanning attempts. Digestion was stopped with 30% FBS. The FBS solution was used to flush and dislodge OPCs on the panning plate with a P1000 pipette tip. The solution was flushed around the rim of the plate towards the centre and again along the edge of the
entire dish, whilst trying to avoid creating too many bubbles in the process. The resulting cell suspension was transferred to a fresh tube. In practice, this stage was difficult to execute due to how strongly the cells were adhering to the plate. Although the initial concentration of adherent cells was not as high as expected when monitored under the microscope, the drop in yield when dislodging cells from the plate was significant at this stage.

The resulting cell suspension was centrifuged at 1200rpm for 15 min at RT and the supernatant was discarded. Due to the low concentration of cells, the cell pellet was resuspended in 400 μl of OPC-GM and seeded into a Sarstedt Cell+ T25 flask. It was allowed to incubate for 7 min at 37°C so that OPCs could adhere and then 4.6 ml of warmed OPC-GM was gently added and returned to the incubator.

**2.2.7 OPC PURIFICATION BY MIXED GLIA SHAKE-OFF**

Primary mixed glia cultures were isolated from neonatal Sprague Dawley rat cortex following a modified standard protocol (Chen et al., 2007, R.Reynolds personal communication). Briefly, P2-P4 Sprague-Dawley rat pups were sacrificed by decapitation and the cortex is dissected. Meninges are removed on sterile filter paper and the cortical tissue is chopped for 5 minutes into ~1mm³ chunks in 1X Trypsin-EDTA and DNase I (Fig. 2.8). The tissue suspension was transferred to a standard T75 flask and placed on an orbital shaker for 20 minutes at 60-80 rpm at 37°C.

![Figure 2.8](image)

**Figure 2.8.** Rat cortical tissue is chopped into 1mm³ pieces and digested in trypsin at 37°C.

Enzymatic digestion was stopped with an equal volume of 10% FBS in DMEM and allowed to shake for a further 5 minutes. The suspension was centrifuged at 1200 rpm for 10 minutes and the pellet was carefully resuspended in 10% FBS DMEM
and plated into T75 flasks (one flask per brain). Cells were cultured for 7-10 days in DMEM supplemented with 10% FBS, 1% pen-strep, and 4 mM glutamine, and kept under a humidified atmosphere at 37°C and 5% CO₂ until confluent. Oligodendrocyte precursor cells (OPCs) were subsequently isolated using a step-based shake-off protocol. Firstly, microglia were depleted by shaking flasks for 1 hour at 200 rpm. Medium was replaced and flasks were shaken overnight at 200 rpm. The medium was collected and transferred to a new flask and incubated for 25 minutes to further deplete adhering microglia. Remaining cells in suspension were resuspended in OPC base medium (DMEM, 1% N2, 1% pen-strep, 4 mM L-glutamine, 100 µg/ml BSA, 63 µg/ml N-acetylcysteine) supplemented with 10 ng/ml PDGF and 10 ng/ml bFGF, or alternatively with 25% B104-conditioned medium. B104-conditioned medium was found to promote the expansion of oligospheres (Zhu et al., 2014). Half the volume of cell culture medium was replaced every two days and was supplemented daily with PDGF/FGF. To induce differentiation, cells were cultured in PLL and laminin-coated flasks in OPC base medium supplemented with 0.5% FBS, 30ng/ml T3 and 400ng/ml T4.

2.2.8 CULTURE OF OPCS AND O2A-MYC CELLS

Protocols for reagents and solutions for the culture of O2A-myc cells and OPCs are detailed in Appendix 2.1.

2.2.8.1 Recovery of O2A-myc cells
Cryovials of O2A-myc cells were recovered from liquid nitrogen storage and quickly warmed at 37°C. Once thawed, the cells were resuspended in 10% FBS, 1% P/S in DMEM and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the cells were gently resuspended in 5ml of OPC/O2A-GM (see Appendix) and seeded into a Cell+ T25 flask.

2.2.8.2 Splitting and freezing of cells
O2A-myc and OPCs were trypsinised gently by removing half of the overlying media and then adding one tenth of the volume of 10x trypsin without EDTA (for instance, if 2ml of medium remained, 200µl of trypsin was added). After 5 min incubation at 37°C, the cell suspension was checked under a microscope and an equal volume of SD mix was then added to the flask to stop enzymatic activity. Cells were centrifuged at 1200rpm for 5 min. Cells were split 1 in 3 at each passage.
High-cell densities were avoided as it induced branching of processes. Alternative cells were resuspended in freezing medium consisting of 90% FBS and 10% DMSO and distributed into labelled cryovials. Cryovials were placed in a Mr. Frosty at -80°C overnight and then transferred to liquid nitrogen for long-term storage.

2.2.8.3 *Coating of cell culture surfaces for OPCs*

Tissue culture plates, glass chamber slides, or glass coverslips were coated with 50 µg/ml poly-lysine diluted in sterile water for 1-2 h, washed twice with sterile water and coated with 10 µg/ml laminin for at least 30 min (see Appendix 2.1 for preparation of stocks). Immediately before use, laminin was removed and the surface was washed one time with PBS.

2.2.8.4 *Generation of B104-conditioned medium (B104CM)*

B104 cells were grown in DMEM (D6429) with 10% heat-inactivated FCS. Cells were split 1 in 6 into T175 flasks. When cells are 50 - 60% confluent, the medium is removed and cells are washed with 30ml PBS. 50ml of DMEM-BS is then added per T175 flask. Supernatant is collected 2-3 days later. The supernatant from all flasks is mixed and homogenised and then centrifuged at 1500 rpm for 10 min to remove any cellular debris. The supernatant is carefully removed and distributed into 33ml aliquots in 50ml labelled tubes and store at -80°C (each aliquot is sufficient to supplement 100ml DMEM-BS). Before use, thaw and centrifuge the B104-CM at 5,000rpm for 10 min in order to remove a precipitate which forms when the conditioned medium is frozen. Filter through a 0.2µm filter and add to 100ml DMEM-BS (25%).

2.2.8.5 *siRNA experimental protocol preparation*

Sterile and RNAse-free conditions were used throughout the siRNA experimental protocol. The vials of Stealth RNAi reagents were resuspended in DEPC-treated water to make a 20µM solution. OPCs were expanded in growth medium, trypsinised and mixed before being seeded in triplicate T25 flasks per treatment group. Flasks were randomised prior to treatment using random.org. Experimental treatment began 24 hours after seeding. Care was taken not to introduce air into the solutions while mixing. RNase-free tubes were labelled and filled with 1x siRNA buffer and media. The appropriate volume of RNA annealing buffer (supplied with the scrambled negative control siRNA) was administered to labelled tubes for 10µM
scrambled siRNA and ATF6 cocktail according to manufacturer’s instructions. The appropriate volume of siRNA stock was added to tubes after it was thawed, vortexed and centrifuged (Fig. 2.89 Table 2). 1.5 ml RNase-free tubes were labelled for scrambled siRNA and ATF6 cocktail at 10nM, 1nM and 0.5nM. 2.0 ml Rnase-free tubes were labelled for RNAiMAX Lipofectamine scrambled siRNA and ATF6 siRNA cocktail for each concentration. Each tube was filled with appropriate volume of serum-free media (Fig. 2.9, Table 1). The relevant volume of siRNA was added to their respective tubes and mixed gently. Tubes were allowed to incubate at room temperature for 5 minutes. Meanwhile, Lipofectamine tubes were filled with appropriate volume of Lipofectamine reagent, mixed gently and allowed to incubate at room temperature for 5 min. The contents of Tube 1 were added to its respective Tube 2 and mixed gently (1:1 ratio) and allowed to incubate for 10-20 min at room temperature. Solutions were mixed again gently before adding 500μL to 4.5ml of cell culture medium containing 0.1μg/ml tunicamycin (1 in 10 dilution) to the relevant T25 flasks to be treated. Flasks were rocked gently back and forth and returned to the incubator for 6-8 hours.

**Table 1**

<table>
<thead>
<tr>
<th>Final siRNA conc</th>
<th>Group</th>
<th>No. T25 Flasks</th>
<th>Total vol diluted siRNA (μL)</th>
<th>Serum free media (μL)</th>
<th>Total serum free media (μL)</th>
<th>Lipofectamine Reagent (μL)</th>
<th>Total vol Lipofectamine (μL)</th>
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<td>0</td>
<td>250</td>
<td>0</td>
<td>825</td>
<td>0</td>
</tr>
<tr>
<td>10nM scrambled</td>
<td>scRNA</td>
<td>3</td>
<td>245</td>
<td>16.5</td>
<td>808.50</td>
<td>825</td>
<td>19.68</td>
</tr>
<tr>
<td>ATF6 cocktail</td>
<td>scRNA</td>
<td>3</td>
<td>245</td>
<td>16.5</td>
<td>808.50</td>
<td>825</td>
<td>19.68</td>
</tr>
<tr>
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<td>1.45</td>
<td>823.35</td>
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<tr>
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<td>1.45</td>
<td>823.35</td>
<td>825</td>
<td>1.96</td>
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<tr>
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<td>0.825</td>
<td>824.18</td>
<td>825</td>
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<tr>
<td>ATP6 cocktail</td>
<td>scRNA</td>
<td>9</td>
<td>0.25</td>
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<td>824.18</td>
<td>825</td>
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**Table 2**

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<th>Serum free media (μL)</th>
<th>Total serum free media (μL)</th>
<th>Lipofectamine Reagent (μL)</th>
<th>Total vol Lipofectamine (μL)</th>
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<td>16.5</td>
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<td>825</td>
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<td>scRNA</td>
<td>3</td>
<td>245</td>
<td>16.5</td>
<td>808.50</td>
<td>825</td>
<td>19.68</td>
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<td>823.35</td>
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<td>823.35</td>
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<tr>
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<td>0.25</td>
<td>0.825</td>
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<td>825</td>
<td>0.98</td>
</tr>
<tr>
<td>ATP6 cocktail</td>
<td>scRNA</td>
<td>9</td>
<td>0.25</td>
<td>0.825</td>
<td>824.18</td>
<td>825</td>
<td>0.98</td>
</tr>
</tbody>
</table>

**Figure 2.9.** Volumes of reagents used for siRNA experimental protocol.
2.2.9 ORGANOTYPIC BRAIN SLICE CULTURES

2.2.9.1 Brain Slice Culture Medium (BSCM)

BSCM was composed of 50% (v/v) Basal Medium Eagle, 25% (v/v) heat-inactivated horse serum, 22% (v/v) Hank’s Balanced Salt Solution, 1% (v/v) penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively), 1% (v/v) L-glutamine, 1% (v/v) N-acetylcysteine and 5mg/ml glucose, sterile filtered through 0.2μm filters into 50ml tubes. This was stored at 4°C.

2.2.9.2 Preparation of Organotypic Brain Slice Cultures

Organotypic cerebellar slice cultures were prepared according to modified protocols from Anna William’s lab, (University of Edinburgh) and published methods (Stopini, Buchs and Muller, 1991; Gähwiler, Thompson and Muller, 1999; Birgbauer, Rao and Webb, 2004; Zhang et al., 2011). Sprague-Dawley rat pups at postnatal day P10 were sacrificed by decapitation. Cerebellar slice cultures were prepared from one pup at a time. The head was dipped in 70% IMS and dissection took place under sterile conditions in a biosafety cabinet with autoclaved instruments. The calvaria was removed and the forebrain was separated from the hindbrain. The cerebellum was dissected as described above and oriented on a sterile silicone platform with a curved forceps on the stage of a McIlwain tissue chopper (GaLa Instrumente, Bad Schwalbach, Germany). Excess liquid on the tissue was removed and the sterile blade was wetted with BSCM before initiating chopping of 400μm thick parasagittal slices. The slices were transferred onto a wet spatula and placed in a petri-dish containing ice-cold sterile-filtered brain slice culture medium (BSCM). Cerebellar slices were separated using #11 scalpel blades under a dissecting microscope. Pasteur pipettes had been prepared with large smooth apertures using a sterile blade to allow easy transfer of whole slices undamaged. Brain slices were cultured on Millicell-CM culture plate inserts (3 slices per insert) (Millipore, Zug, Switzerland) in 6 well tissue culture plates and incubated for 7 days at 37°C in a humidified atmosphere with 5% CO₂ before experiments began. Medium was changed three times a week. Stained organotypic cerebellar slices showed axons were myelinated in culture (Fig. 2.10).
Figure 2.10. Confocal z-stack image of organotypic cerebellar slice culture after 14 days in vitro. Calbindin (green) labels Purkinje cell axons. The mature myelin marker MOG (red) can be observed surrounding axons in white matter tracts in the xz and yz planes.

2.2.9.3  Demyelination of Cerebellar Slices

Lysolecithin, also known as lysophosphatidylcholine (LPC), is used as a detergent to analyse membrane processes (Weltzien, Richter and Ferber, 1979) (Fig. 2.11). Though its specific effects on demyelination are not completely understood, its membrane-solubilising properties are particularly toxic to oligodendrocytes in vitro and in vivo (Woodruff and Franklin, 1999a; Woodruff and Franklin, 1999b). It may also cause cell loss of astrocytes and neurons in serum-free media (Defaux et al., 2010).

Figure 2.11: Chemical structure of lysolecithin, or its IUPAC name (2-\{3-\{10-[(1S,2R)-2-Hexylcyclopropyl]decanoyl\}oxy\}-2-hydroxypropylphosphonato\}oxy\}ethyl) trimethylazanium.
Lysolecithin was reconstituted to a concentration of 10mg/ml in sterile H₂O, aliquoted and stored at -20°C. Vials were thawed and added to BSCM in 0.1 - 0.5mg/ml final concentrations. Slices were incubated with LPC for 15-17 hours and then transferred to a new well with fresh medium (Birgbauer, Rao and Webb, 2004; Zhang et al., 2011). Peak demyelination occurs within 48 hours and remyelination can be detected at 14 days post LPC (Birgbauer, Rao and Webb, 2004).

2.2.10 STATISTICAL ANALYSIS
Data were analysed using GraphPad Prism Version 5.0 software. Statistical analyses were carried out using Kruskal-Wallis tests with multiple-paired comparisons for non-parametric data or One/Two Way ANOVA with Bonferroni multiple-comparison analysis for parametric data, as appropriate. Data are expressed as mean and the standard error of the mean (SEM). When statistical analysis was performed on qPCR data, mean Ct values were used for analysis and graphs were shown as fold change. Differences were deemed statistically significant if \(p < 0.05\).
CHAPTER 3

PROFILE OF THE UNFOLDED PROTEIN RESPONSE IN RAT CEREBELLAR CORTICAL DEVELOPMENT
3.1 INTRODUCTION

3.1.1 Cerebellum

The elegant, geometric arrangement of the cerebellar cortex has been admired since first described by the earliest neuroanatomists (Purkyne, 1838; Ramon y Cajal, 1911). Its relatively simple circuitry, compact, modular structure and stereotyped development held great promise that the cerebellum could help unlock mechanisms of CNS development, injury and disease (Eccles, Ito and Szentágothai, 1967). To the present day, the cerebellum has been used as a basis for studies of neuronal histology, maturation, electrophysiology, circuit assembly, and myelination in particular. However, no sooner had it been claimed by Eccles that “all Purkinje cells are functionally homologous”, that the complex compartmentalisation of the cerebellum was becoming evident (Voogd, 1967; Armstrong, Harvey and Schild, 1974). This field was reawakened with the discovery of zebrin II patterning which has since been succeeded by a plethora of other molecules that have redefined the cerebellum as an assembly of non-uniform PC networks (Cerminara et al., 2015). PCs are the first neurons to reach their destinations in the cerebellar cortex and give cues to immature mossy fibres, climbing fibres, GCPs, interneurons and neural stem cells to aid their appropriate differentiation in time and space (Sotelo, 2004; Apps and Hawkes, 2009; Consalez and Hawkes, 2013; Fleming et al., 2013a). Hence they are crucial in the construction of the entire cytoarchitecture of the cerebellar cortex. Striping topography is an evolutionarily-conserved and life-long feature of PCs that is rapidly established after their formation. Moreover, this molecular identity of PCs is intransigent to manipulation. PCs commit to a specific molecular profile as early as E14.5 and retain this identity for life, regardless of elimination of neuronal activity or afferent fibre input (Leclerc, Gravel and Hawkes, 1988; Seil, Johnson and Hawkes, 1995; Armstrong et al., 2001a; Larouche and Hawkes, 2006). Organotypic coronal cerebellar slices maintain parasagittal striping topography in culture after 10 days in vitro (DIV) (Furutama et al., 2010). Non-uniform expression is also observable in lobes of sagittally-oriented sections and slice cultures (Armstrong et al., 2000; Armstrong et al., 2001b; Gincel et al., 2007).

Some striping markers, such as zebrin II/aldolase C, extend the entire medial lateral axis of all lobules; others, such as HSP25, are relatively more simple. Striping is absent in the anterior zone and only subtly demarcated in transverse and coronal sections of
the posterior and nodular zone. In contrast, a sagittal section of the vermis would show robust expression along select lobules that disappears in subsequent sections (Fig 3.1).

Figure 3.1. The constitutive expression pattern of HSP25 in mouse cerebellum viewed in coronal and sagittal orientation. The relative rostrocaudal positions of transverse sections (A–G) are indicated on the drawing of a sagittal section through the midline (H) which outlines the extent Hsp25 expression domains in the vermis in bold. Abbreviations: PfS, parafloccular sulcus; PIF, posterolateral fissure; spV, spinal trigeminal tract. Scale bar = 500 μm applies to A–G. Image from (Armstrong et al., 2001a).

For this reason, the possibility of missing, or indeed dismissing, potentially important findings is considerable. Functional electrophysiological properties of PCs also vary according to zebrin patterning (Ebner et al., 2012; Zhou et al., 2014). Because firing
patterns are present in organotypic slices (Kessler et al., 2008; Mordel et al., 2013), and the fact that there is now an established link between myelination and electrical activity (Gibson et al., 2014), this added complexity emphasises the need for randomisation and other due considerations in using cerebellar explants as an experimental model. Preferential survival of parasagittal PC subsets (Zebrin II/EAA4-positive) has been reported for a range of insults including trauma and excitotoxicity (Slemmer et al., 2007), ischemia (Welsh et al., 2002) and neonatal Borna disease (NBD) virus infection (Williams et al., 2007). Neurodegeneration in NBD not only discriminates between zebrin II/EAA4-expressing PC subsets, but also leads to banded expression of metallothionein-I/II and CD44 in astrocytes and dense bands of microglia in the ML of zebrin-negative zones (Williams et al., 2007), i.e. patterning effects on glial cells not classically associated with parasagittal striping topography. Interestingly, this group failed to make these observations in their first study of cerebellar neurodegeneration and microglia activation in NBD which had not considered this topography during analysis (Weissenböck et al., 2000). This highlights the need for awareness of cerebellar compartmentalisation and its potential consequences. Increasing the relevance to our studies is the fact that the UPR has been implicated in NBD-induced neurodegeneration, with increased expression of CHOP and ATF4 observed in zebrin II-positive PC subsets at the height of disease (Williams and Lipkin, 2006).

While an evolutionarily-conserved, molecular, sagittal striping pattern in the cerebellum is now well-recorded in the literature, its bearing and relevance on model systems that use the cerebellum would appear to be neglected.

3.1.2 The Unfolded Protein Response in cerebellar cortex

The Unfolded Protein Response (UPR) is evoked by stress to the endoplasmic reticulum (ER stress). When ER processing capacity is compromised, misfolded proteins begin to accumulate and can activate ER stress sensors (PERK, IRE1, ATF6) to initiate the UPR. This highly-conserved homeostatic response initially blocks translation of most proteins via peIF2α so that immediate pressure on the ER is alleviated. UPR transcription factors (ATF4, XBP1s, ATF6) upregulate chaperones (such as GRP78, GRP94 and PDI) to directly resolve protein misfolding, as well as genes that increase ER size and processing capacity. The UPR is dynamic and
adaptable to changing severity and duration of ER stress and will induce apoptosis if the stress cannot be surmounted.

Neurons have an extensive ER network to facilitate calcium storage and signalling. In PCs, it comprises a massive continuous compartment throughout the dendrites, cell body and axon (Terasaki et al., 1994) and it is postulated that neurons in general may be especially susceptible to ER stress. Indeed Alzheimer’s, Parkinson’s, Huntington’s disease, prion disease, amyotrophic lateral sclerosis and fronto-temporal dementia are all characterised by aggregations of misfolded proteins and all show markers of an activated UPR (Schepers and Hoozemans, 2015; Torres et al., 2015).

The UPR is activated during embryonic CNS development and in the specification of cortical neurons (Zhang et al., 2007; Laguesse et al., 2015). A developmental role for the UPR has been postulated for the cerebellum based on cerebellar malformations due to defective ER chaperone function (Marzban et al., 2015). Marinesco-Sjogren syndrome is a genetic disorder associated with mutations in Sil1, a nucleotide exchange factor for GRP78, and causes prominent cerebellar defects. Zhao, et al. showed that the phenotype of Sil1−/− mice could be rescued by overexpression of ER chaperone ORP150, or ameliorated by loss of DNAJ5, a promoter of the GRP78 ATP/ADP cycle (Zhao et al., 2010).

Unfortunately, the majority of studies assess a single arm of the UPR, and all too frequently only 1-2 proteins. For a homeostatic system that shares a common initiation mechanism and synergistic signalling cascades, it is surprising that so few investigations of the UPR assess all three arms in parallel. Much still remains to be learned about the potential role of the UPR in cerebellar development (Marzban et al., 2015).

It is argued that research employing the cerebellum as a model system should be aware of its non-uniform patterned composition and how that may impact analyses. The identification of the chaperone HSP25 as a conserved striping marker is of relevance to this work as the UPR upregulates heat shock proteins GRP78 (HSPA5) and GRP95 (HSP90B1). In addition, the extensive ER compartment of PCs and other neurons, and the potential role of the UPR in patterned degeneration of PC subsets, also gave cause to consider whether ER-resident chaperones or other molecules could exhibit an intrinsic striping pattern. Furthermore, XBP1 staining carried out by a former M.Sc.
student in our group (Orlagh Moran) was suggestive of potential XBP1-positive and XBP1-negative PC populations in the rat cerebellum. It seemed important to ascertain whether any UPR markers of interest exhibit a parasagittal striping pattern. This would clarify whether specific changes in sagittal tissue sections or organotypic slice cultures would be due to experimental manipulation or to intrinsically higher physiological expression in that region.

Therefore, in order to utilise the cerebellum as a model for study of \textit{in vivo} developmental myelination and take advantage of the rich literature that already exists for this model, it appeared necessary to first rule out any potential striping confounds. By screening for UPR molecules of interest, it simultaneously provides the opportunity to contribute a comprehensive profile of UPR signalling which has not been undertaken to date and potentially provide new insights in cerebellar cortical development. In addition, this screening would indicate whether UPR activation is specific or general to various cell types or cerebellar zones at different stages. This could inform drug targeting and other manipulations of this pathway in the CNS in future.

\textbf{3.2 RATIONALE AND HYPOTHESIS:}

Our central hypothesis concerns the potential role of the UPR in myelination. Classical and modern myelination studies have utilised the cerebellum to characterise oligodendrocyte maturation \textit{in vivo} and \textit{ex vivo}. Despite widespread use of the cerebellum as a model for many CNS processes, the complexity of cerebellar cortical organisation is frequently not addressed. This may lead to erroneous reports or delays in accurate interpretation. A potential role has been indicated for the UPR in cerebellar development but a comprehensive profile of this pathway is so far lacking. To this end, we aimed both to screen and characterise the expression profile of UPR markers in the cerebellar cortex during development.

\textbf{Hypothesis:}

The UPR is activated during postnatal development of the rat cerebellum.

\textbf{3.3 AIMS}

\begin{itemize}
  \item Screen UPR proteins of interest for evidence of striping topography in rat cerebellum
\end{itemize}
• Quantify changes in gene expression of UPR markers in cerebellar development

• Assess the activation status of UPR initiators and expression of downstream targets in all cortical layers of the cerebellum during development

Note: The methods for this study have already been described in detail in Chapter 2.
3.4 RESULTS

3.4.1 Wholemount IHC for screening of UPR proteins for evidence of striping topography in rat cerebellum

Adult rat cerebella were dissected, fixed, processed, rehydrated and digested for wholemount IHC according to a published protocol (Section 2.2.3.14, Sillitoe & Hawkes, 2002). Imaging of stained samples was undertaken by an M.Sc. student, Alan Hoban, who was trained and supervised for the titration of zebrin II hybridoma supernatant and detection of ER stress markers. A 1:10 dilution was optimal for detection of positive striping of zebrin II/aldolase C (Fig 3.2).

**Zebrin II/aldolase C**

**Neg. control**

*Figure 3.2.* Wholemount staining of adult rat cerebellum using zebrin II/aldolase C monoclonal antibody. Alternating parasagittal stripes can be observed for zebrin II/aldolase C. Open and closed arrows indicate negative and positive staining
respectively. Negative control (no primary antibody) was absent of non-specific staining. Scale bar is 250 µm.
**Figure 3.3.** Wholemount immunohistochemistry of adult rat cerebella for UPR markers. Positive staining for pIRE1, ATF6, XBP1 is evident. No staining was observed in the corresponding negative control. Scale bar is 250 µm.

Wholemount immunostaining showed positive detection for antibodies against pIRE1, ATF6 and XBP1 in adult cerebellum but they did not show evidence of a patterned topography (Fig. 3.3). Titration of the zebrin II antibody highlighted that under- or over-staining of cerebellum could easily mask striping topography, an effect also seen when using neurofilament antibody (Appendix 3.1). Therefore a more sensitive method was preferred to allow screening for parasagittal striping with more confidence.

### 3.4.2 Screening for evidence of patterning in transverse sections of rat cerebellum

To assess potential patterning of UPR molecules more sensitively and to ensure that any potential striping markers outside of the molecular layer would also be detected, paraffin-embedded transverse sections of adult rat cerebellum were immunostained using the ABC-DAB method. The bilateral symmetry of differentially expressing cells around the vermis would serve as an indicator of potential patterning. Transverse sections of fixed paraffin-embedded adult rat cerebellum were prepared and the M.Sc. student Alan Hoban was trained and supervised in staining and imaging of sections.

Zebrin II/aldolase C expression was observed in positive and negative parasagittal bands in PC bodies and extended throughout PC dendrites in the ML (Fig. 3.4). The alternating positive and negative bands of expression, symmetrically aligned in parasagittal orientation for zebrin II/aldolase C, validated the use of transverse sections for the indication of a patterned topography.
Figure 3.4. Zebrin II/aldolase C striping in the vermis of the cerebellum. (A) Alternating parasagittal bands of zebrin II immunoreactivity were observed in the Purkinje cell layer and molecular layer (indicated by ‘+’ and ‘-’). (B) Digital zoom of positive and negative areas of expression boxed in A.

Subsequent staining of sections with UPR-associated molecules displayed varied patterns of expression in cerebellar cortex (Fig. 3.5). Observable patterns of expression are summarised in Table 3.1. Several markers such as GRP78 and ATF6 showed uniform positive expression in cerebellar cortex (Fig. 3.5), whereas others, such as PDI, were uniformly negative. Only pIRE1 and XBP1 displayed non-uniform staining
of Purkinje cells (Fig. 3.5). False positive staining of Purkinje cells was detected in negative primary antibody controls using ABC-DAB IHC (Fig. 3.5). This was likely due to the presence of endogenous biotin in PCs which could be bound by streptavidin-biotin HRP complexes during staining (McKay, Molineux and Turner, 2004).

**Figure 3.5.** Immunoreactivity of UPR-associated markers in transverse sections of the vermis of adult rat cerebellum using ABC-DAB IHC. Open and closed arrows indicate negative and positive staining respectively. GRP78 antibody uniformly stained PCs and other neurons in the cerebellar cortex. Positive ATF6 staining was uniform in ML of all regions. pIRE1 and XBP1 expression was non-uniform in PCs in all regions. False positive staining present in PCs of negative primary antibody control tissue.

An alternative antibody detection method, using alkaline phosphatase and liquid permanent red as chromogen, was employed to circumvent the labelling of
endogenous biotin. This staining replicated the differential expression of pIRE1 and XBP1 in Purkinje cell bodies whilst eliminating false-positive signal of PCs in negative controls (Fig. 3.6). A symmetrical pattern of expression could not be established however for either pIRE1 or XBP1. As summarised in Table 3.1, only zebrin II/aldolase C displayed positive expression in a symmetrical, alternating pattern around the vermis in the rat cerebellum sections. This, in conjunction with the lack of detectable patterning in wholemount-stained cerebella, allowed us to conclude that UPR-associated proteins examined were not present in the alternating pattern of expression typified by zebrin II/aldolase C.

![Figure 3.6. Immunoreactivity of pIRE1 and XBP1 in transverse sections of the vermis of adult rat cerebellum using AP-LPR IHC. pIRE1 and XBP1 showed heterogeneous expression in PCs in all regions. Background staining was eliminated in negative primary antibody control tissue.](image-url)

Figure 3.6. Immunoreactivity of pIRE1 and XBP1 in transverse sections of the vermis of adult rat cerebellum using AP-LPR IHC. pIRE1 and XBP1 showed heterogeneous expression in PCs in all regions. Background staining was eliminated in negative primary antibody control tissue.
Table 3.1. Summary of staining patterns detected for antibodies against zebrin II/aldolase C and UPR molecules in transverse sections of adult rat cerebellum.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Expression</th>
<th>Alternating</th>
<th>Symmetry</th>
<th>Striping</th>
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<tr>
<td><strong>Zebrin II/aldolase C</strong></td>
<td>Positive in PCL and ML</td>
<td>Yes</td>
<td>Yes</td>
<td>✓</td>
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<tr>
<td>pPERK</td>
<td>Negative</td>
<td>No</td>
<td>N/A</td>
<td>X</td>
</tr>
<tr>
<td>pIRE1</td>
<td>Positive in PCL</td>
<td>Yes</td>
<td>No</td>
<td>X</td>
</tr>
<tr>
<td>ATF6</td>
<td>Positive in ML</td>
<td>No</td>
<td>N/A</td>
<td>X</td>
</tr>
<tr>
<td>peIF2α</td>
<td>Negative</td>
<td>No</td>
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<td>X</td>
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<td>CHOP</td>
<td>Negative</td>
<td>No</td>
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<td>XBP1</td>
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3.4.3 Gene expression profile of UPR signalling in developing cerebellum

To determine whether genes classically associated with a UPR response were altered during development, gene expression was assessed by qPCR in P7, P10, P14, P17 and adult cerebellum. Activated IRE1 and ATF6 regulate UPR transcriptional programmes through spliced XBP1 and cleaved ATF6 transcription factors, respectively. Quantitative PCR analysis revealed dramatically increased mRNA expression of spliced XBP1 between P7 and adult (approx. 150-fold). ATF6 gene expression also increased significantly between P10 and P17 (Fig. 3.7A). However, gene expression of UPR targets GRP78, GRP94 and PDI did not change significantly over time (Fig. 3.7B).
Figure 3.7. UPR gene expression in developing rat cerebellum. (A) mRNA expression of spliced XBP1 increases dramatically between P7 and adult. ATF6 mRNA is significantly increased between P10 and P17. (B) mRNA expression of UPR targets GRP78, GRP94 and PDI show no significant change over time. Data are expressed in $2^{\Delta \Delta CT}$. One-Way ANOVA with Tukey post test (n=3). *p<0.05.
3.4.4 Initiators of UPR signalling are differentially activated during cerebellar cortical development

To determine whether changes in UPR signalling were specific to different cortical layers, tissue sections were stained and analysed with respect to individual GM layers during cortical development. Sagittal sections allowed clear delineation of all cerebellar layers within individual lobules, for accurate anatomical positioning.

ATF6 expression was low or negligible in most regions at P10, P14 and adult and was notably absent in mature large neurons (PCs, Golgi cells, cerebellar nuclei) (Fig. 3.8 and 3.9 and Table 3.2). At P14, ATF6 staining became conspicuous within cells having the morphology and distribution typical of oligodendrocytes in WM and GM and Bergmann glia astrocytes. This pattern became more prominent in adulthood (Fig. 3.9). The specificity of increased ATF6 expression to these populations is notable in non-counterstained tissue (Fig. 3.10B). ATF6 cells in the WM, IGL, and ML displayed the same relative spatial distributions as those of olig2-positive cells in respective regions (Fig. 3.10C). ATF6-positive cells were also observed forming oligodendrocyte “chains” (Fig. 3.10, left insets). Although Bergmann glia were well-defined by GFAP staining at P10, ATF6 expression was observed only in this population from P14. ATF6 positivity was also observed in adult Bergmann glia surrounding Purkinje cell bodies and tracing processes extending towards the glia limitans (Fig. 3.10, right insets in A and D).

Figure 3.8

ATF6 expression in the deep cerebellar nuclei. ATF6 was absent in mature large neurons of the DCN but was strongly expressed in glia with the morphology of mature oligodendrocytes.
Figure 3.9. ATF6 expression in the developing cereellar cortex. ATF6 expression was associated with Bergmann glia in the ML. Scale bar indicates 50 µm.
A. ATF6

B. ATF6

C. Olig2

D. GFAP
Figure 3.10 (previous page). ATF6 immunostaining detected cells with similar morphology and distribution to oligodendrocytes and Bergmann glia in adult rat cerebellum. (A) and (B) show ATF6 expression in counterstained and non-counterstained tissue respectively. (C) Olig2 expression closely matches relative distribution of ATF6-positive cells in WM and GM layers. ATF6 expression can be observed forming oligodendrocyte “chains” similar to olig2 staining (asterices, left insets in A-C). (D) Bergmann glia stained with GFAP match the morphology of ATF6 positive cells observed surrounding Purkinje cell bodies and extending towards the glia limitans (arrows, right insets in A and D). Scale bar indicates 100 µm.

In contrast to ATF6, pIRE1 staining was notable in mature large neurons of the BS, DCN, ML, PCL and was abundant in all layers (Fig. 3.11 and Table 3.2). Strong immunoreactivity was noted in a subset of granule cell precursors in the EGL which was lost upon addition of blocking peptide (Fig. 3.12). Some tissue blocks were prone to increased background when stained with this antibody including acellular regions in the ML. Unfortunately, efforts to titrate this antibody to reduce background resulted in loss of cellular staining. The addition of blocking peptide showed that the antibody-antigen reaction in cellular staining was specific to pIRE1 and the remaining low background colouration was the result of non-specific staining arising from the staining protocol (Fig. 3.12).
Figure 3.11. pIRE1 expression in the developing cerebellar cortex. Activation of IRE1 was abundant in neurons of the ML and PCL (see Table 3.1). pIRE1 was also present in a subset of granule cell precursors in the EGL. Scale bar indicates 50 µm.
Figure 3.12. Incubation with blocking peptide eliminates positive pIRE1 staining in the EGL. Staining of the EGL for pIRE1 with conventional IHC protocol (left) is compared to sections treated with antibody incubated with blocking peptide (right). Peptide-blocking prior to IHC resulted in loss of cellular staining. Scale bar indicates 50\(\mu\)m.

A population of pPERK-positive cells was prominent in a subset of precursors in the EGL and in a potentially migratory cell population distributed throughout the PWM, IGL and ML in postnatal tissue. These cells were evenly distributed across all lobules and could be observed in the periphery of the DCN at P7 but were located progressively farther into the upper reaches of the lobules as time progressed. This distinctive population appeared to decrease in parallel with the diminishing EGL and no positive cells were observed in adult GM layers (Fig. 3.13). The addition of blocking pPERK peptide showed the staining of cells to be specific (Fig. 3.14).
Figure 3.13. pPERK expression in the developing cerebellar cortex. This antibody detected darkly-stained cells in postnatal PWM, IGL, ML and EGL but these were not present in adult tissue. Scale bar indicates 50 µm.
The distribution of positive pPERK cells was similar to the population positive for pIRE1 at P7 (Fig. 3.15 and 3.16). Actively dividing positive cells were frequently observed in the EGL and occasionally in the PWM for pPERK and pIRE1 at P7 (Fig. 3.15 C, D). Transverse sections of cerebellar tissue allow distinction between the proliferative zone and the differentiation zone of GCPs within the EGL. Positive cells for pPERK or pIRE1 were both predominantly located in the outer proliferative zone (Fig. 3.17). pIRE1-positive cells were not quantified due to its broad expression in more mature tissue (Fig. 3.11) but the specificity of pPERK staining facilitated quantification within individual GM layers. The density of pPERK-positive cells was found to significantly decrease between P7 and P17 in the PWM, IGL and EGL (Fig. 3.18). Very low numbers were observed in the ML and there were no significant differences in the ML over time.

**Figure 3.14.** Incubation with blocking peptide eliminates positive pPERK staining in the EGL. Staining for pPERK with conventional IHC protocol (left) is compared to sections treated with antibody incubated with blocking peptide (right). Peptide-blocking prior to IHC resulted in loss of cellular staining. Scale bar indicates 50μm.
Figure 3.15. pPERK and pIRE1 stain similar populations of cells in P7 rat cerebellum. (A) pPERK is expressed in a subset of granule cell precursors and an unidentified population in PWM and IGL/ML. Positive cells are indicated with *. (B) pIRE1 expression is detected in a similar subset of cells at P7 but this subgroup is not observable at later timepoints due to broad pIRE1 expression in more mature tissue. Positive cells are indicated with *. (C) Insets from A and B show similarity of cells detected for positive pPERK and pIRE1 expression in the EGL, PWM and IGL. Actively dividing cells in metaphase and anaphase were frequently observed in the EGL (C) and occasionally in the PWM (D). Scale bar indicates 50μm.
Figure 3.16. pPERK and pIRE1 stain similar populations of cells in P10 rat cerebellum. pPERK staining gave strong, robust signal which facilitated cell counting whereas pIRE1 staining was more prone to artefact (note edge effect) and its broad expression in the parenchyma at older ages made cell counting prohibitive. Scale bar indicates 500μm.
Figure 3.17. Transverse sections of rat cerebellum at P5 stained for pPERK and pIRE1. The majority of positive cells reside in the upper proliferative zone (pz) rather than the differentiation zone (dz) of the EGL for both pPERK (left) and pIRE1 (right). Scale bar indicates 50 μm.

Figure 3.18. Quantitative assessment of the density of pPERK-positive cells in cerebellar cortical layers (EGL, ML, IGL and PWM) at P7, P10 and P17. Kruskal-Wallis statistical tests were applied and Tukey-Kramer post hoc tests showed where significant differences lay between age groups (n=3). * denotes $p<0.05$, ** $p<0.01$. 
3.4.5 *Downstream targets of the UPR are differentially expressed in rat cerebellar cortex*

Sections were then stained to detect whether the presence of activated UPR initiators was accompanied by increased expression of UPR targets. Phosphorylation of eIF2α and CHOP are classically-associated downstream events of PERK signalling. No positive staining was observed for p-eIF2α and detection of CHOP was also negative at all timepoints bar faint glomerular staining in adult GL (Fig. 3.19).

![Figure 3.19. CHOP staining is negative at P7 (left) and adult (middle) cerebellum bar faint glomerular staining in the mature GL (right). Scale bar indicates 200μm.](image)

The activation of IRE1 enables splicing of cytosolic XBP1 mRNA which leads to translation of the XBP1s transcription factor and its translocation to the nucleus. Sections stained for XBP1 showed variable background which prevented reliable evaluation. Chaperones and foldases, GRP78, GRP94 and PDI are constitutively expressed proteins in the ER but their upregulation is a classical signature of UPR activation. Detection of GRP78 was abundant in all regions at all timepoints but staining was more intense as the cerebellum matured (Fig. 3.20). GRP94 expression followed a near-identical pattern (Fig. 3.21). Both chaperones were strongly expressed by large neurons including Purkinje cells, Golgi cells and neurons of the DCN. In contrast, PDI was much less frequently expressed and largely absent in neurons but was associated with Bergmann glia at early timepoints (Fig. 3.22).
Figure 3.20. ER chaperone GRP78 was notably expressed by Purkinje cells, Golgi cells, stellate and basket cells as well as large neurons in cerebellar and brainstem nuclei. B=basket cell, S= stellate cell, G= Golgi cell. Scale bar indicates 50 µm.
Figure 3.21. GRP94 was strongly expressed by Purkinje cells, Golgi cells and other neurons of the cerebellar cortex similar to GRP78. B=basket cell, S=stellate cell, G=Golgi cell. Scale bar indicates 50 µm.
Figure 3.22. PDI, a redox enzyme of the ER, was associated with Bergmann glia during development but was not frequently expressed in the cerebellar cortex compared to GRP78 and GRP94. BG=Bergmann glia. Scale bar indicates 50 µm.
3.4.6 *Summarised expression profile of the UPR in the developing cerebellar cortex*

To summarise these observations, the abundance of positive cells for each protein was semi-quantitatively assessed in sections from a minimum of n=3 animals. Expression was categorised according to apparent abundance of positive cells in that region, regardless of the intensity of staining, and summarised in Table 3.2.

**Table 3.2.** Expression profile of initiators and downstream targets of the UPR during cerebellar cortical development.

<table>
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<tr>
<th>Region</th>
<th>Age</th>
<th>pPERK</th>
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<th>ATF6</th>
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<th>GRP94</th>
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Semi-quantitative assessment: 0 - no positive cells observed; 1 – 0-19%; 2 – 20-39%; 3 – 40-59%, 4 – 60-79% positive; 5 – 80-100%. 2nd Pw = P7-P10 timepoints. 3rd Pw = P14-P17 timepoints.

It is clear that the expression of activated UPR signalling pathways can be different for a given region or timepoint and indeed can be highly cell-specific, e.g., expression of ATF6 in Bergmann glia which was not accompanied by pPERK or pIRE1. pIRE1 was highly abundant in most cortical layers and conspicuous in large neuronal cell bodies. A similar pattern was observed for GRP78 and GRP94 chaperone expression.
In contrast, PDI expression was much less frequent and only transiently associated with Bergmann glia in development.

Encouragingly, this IHC expression profile would appear to be in agreement with respective mRNA levels of whole cerebellum (Fig. 3.7). Both indicate higher overall ATF6 expression in more mature tissue and no significant changes in levels of GRP78, GRP94 or PDI in the cerebellum over time. Although IRE1 had not been quantified by qPCR, presence of XBP1s mRNA is considered a direct downstream event of IRE1 activation and these both showed overall increases over time (Table 3.2 and Fig. 3.7A).

3.5 DISCUSSION

This study had three primary aims: 1) to screen markers of the UPR for the presence of parasagittal topography; 2) to quantify UPR-related gene expression changes in cerebellar development and 3) to compile a developmental profile of UPR signalling in developing cerebellar cortical layers. Given that the UPR has been implicated in several reports of parasagittally-aligned cell death in the cerebellum it was important to establish whether intrinsically higher UPR signalling could be associated with PC subsets in physiological settings. To address the first of these aims we employed wholemount immunohistochemistry. This method has been published as a tool for high-throughput screening to identify striping topography in the cerebellum and has been successfully applied across 23 different species (Sillitoe and Hawkes, 2002; Sillitoe et al., 2005). However, in practice, this 10 day protocol could not be employed in a high-throughput manner and required a large number of animals in conflict with the principles of the three Rs. Switching to IHC of paraffin-embedded transverse sections was a more sensitive and reliable method that facilitated faster screening with fewer animals compared to wholemount IHC which required one animal per stain. Initiators and downstream targets of all three arms of the pathway were screened and the hallmarks of striping topography (alternating positive and negative bands symmetrically aligned around the vermis) were not observed for any protein of interest.
Having determined that none of our proteins of interest exhibited a striped pattern in parasagittal zones, a developmental study examining the profile of the UPR in cerebellar cortex and cerebellar white matter (Chapter 4) could be carried out with confidence using sagittal sections. This analysis of the cerebellar cortex focused on critical postnatal phases in circuit formation and GM stratification. PCs and interneurons of the cerebellum only reach the cortex at the end of the first postnatal week. In the second postnatal week (2nd Pw) the cell bodies of PCs swell and extend dendrites into the ML. Basket cell synapses form on PC somata before their ensheathment by Bergmann glia and PC dendrites establish contacts with parallel fibres (Altman, 1982). Meanwhile the EGL thickens to a maximum depth at P10 and begins to wane thereafter. Extensive refinement of cortical circuitry occurs in the third postnatal week including the elimination of excess climbing fibres through synaptic competition and further migration and maturation of GCPs until the egress of the EGL is fully complete at P21.

This study showed abundant activation of IRE1 in the DCN, PCL and ML in developing and mature cerebellum which increased overall with time. This increase was accompanied by rising levels of XBP1s mRNA in maturing cerebellum. The presence of IRE1 signalling was not accompanied by activation of PERK or ATF6 signalling in these regions. Differential expression of UPR signalling pathways was also observed in EGL precursors and Bergmann glia. Much of our basic understanding of the UPR has been gleaned from in vitro studies which use inducers of ER stress such as tunicamycin, thapsigargin or dithiothreitol (DTT), which directly disrupt ER luminal protein processing. These rapidly and robustly activate all three arms of the UPR and showed that PERK, IRE1 and ATF6 have a common triggering mechanism via dissociation of GRP78 (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002). It is increasingly apparent that selective activation of ER stress sensors is not unusual in more physiological settings however e.g., in the specification of cortical neurons (Laguesse et al., 2015) and in differentiation of B lymphocytes (Gass et al., 2009; Ma et al., 2010). The mechanisms facilitating selective activation are still being investigated but perturbations in the composition of the ER membrane could play a role. Expression of calcium-independent phospholipase 2 gamma (iPLA2γ) which lies on the ER membrane has been shown to enhance activation of the ATF6 signalling pathway and induction of chaperones (Elimam et al., 2015). ATF6 activation was also
selectively enhanced by the expression of a tail-anchored ER membrane protein (Maiuolo et al., 2011). Mutant PERK and IRE1 transmembrane proteins missing their ER-sensing domains have been shown to be capable of activating a UPR in response to saturated lipid concentrations in the ER membrane independently of protein folding homeostasis (Volmer, van der Ploeg and Ron, 2013). These studies point towards more nuanced and dynamic UPR signalling which may be tailored to stage-specific needs of diverse cell types.

Likewise, differential expression of UPR targets was also observed with a high proportion of GRP78 and GRP94-positive cells in cortical layers but detection of PDI-positive cells was much less frequent. D’Souza and Brown showed constitutive expression of chaperones in developing and adult rat cerebellum and similarly we observed no significant change in mRNA levels of these proteins over time (D’Souza and Brown, 1998). We did not observe positive cells for expression of p-eIF2α or CHOP, which are considered early and late-stage events of canonical PERK signalling. This was surprising considering the robust, persistent and specific activation of PERK in the EGL and PWM. Phosphorylated eIF2α effects a blockade in protein translation and CHOP is traditionally associated with ER stress-induced apoptosis. Potentially these cells could be undergoing peIF2α-independent modifications, possibly associated with autophagy. This follows from recent reports of PERK-dependent, but peIF2α-independent, regulation of autophagy via pAMK, HSP90 or pFOXO-beclin1 (Liu et al., 2015), and the commonly-reported need for functional autophagy in the normal development of cerebellar neurons (Marzban et al., 2014). However further investigation is required to confirm this.

Strikingly, cells positive for activated PERK displayed a subset of GCPs as well as a population of intensely-stained cells in the PWM and other layers at postnatal timepoints. A similar staining pattern was detected for activated IRE1 at P7 but its abundance in other cell types at later timepoints obscured whether this pattern continued.

Indeed the failure to find an effective immunofluorescent staining protocol for these proteins in in vivo tissue sections, (which is also generally absent in the UPR literature), and lack of access to reporter mice, did not permit dual-staining to confirm colocalisation of these proteins in the same cells. Therefore no definitive conclusions
are permitted at this time on whether pIRE1 and pPERK signalling are active simultaneously in the same populations or indeed whether they stain a consistent population over time and which cell types they are associated with.

In the absence of these tools, all propositions are merely exploratory and speculative. Nonetheless, given the high-proliferative activity observed in these cells and the relevance of the EGL and the cerebellar PWM as some of the most common sites of origin for malignant childhood tumours in the CNS, it appears nonetheless important to address how this population compares with studies already taken in this field.

Given the regularity of cell size and staining intensity observed with pPERK-expressing cells and their progressive decrease from inner to outer layers, it was hypothesised whether these cells shared a common lineage or transition event during cerebellar development. As the EGL is composed of GCPs that exclusively generate granule cells which do not enter the PWM at any stage, this suggests that at least two separate populations express pPERK in the postnatal cerebellum. No obvious relationship was apparent between pPERK-positive cells and microglia, astrocytes, oligodendrocytes, Bergmann glia, Purkinje cells or DCN neurons given the relative distribution, cell density and maturity of these cells at each timepoint. Unipolar brush cells are late-developing glutamatergic interneurons of the cerebellum that spread through the PWM and do not fully differentiate until the 4th Pw (Leto et al., 2016). However these cells have a characteristic distribution that is highly concentrated in lobules of the vestibulocerebellum (IX and X) and do not proliferate during their migration in the PWM (Fig. 3.23) (Diño, Willard and Mugnaini, 1999; Englund et al., 2006). In contrast the pPERK-positive population was distributed evenly in all lobules and observed proliferating in the PWM (Fig. 3.24).
Figure 3.23. Localisation of UBCs in the adult mouse cerebellum. Tbr2 positive UBCs are concentrated in lobules IX and X and are relatively rare in the anterior cerebellum. Image source (Englund et al., 2006).

Figure 3.24. Sagittal section of P7 cerebellum shows cells positive for pPERK are distributed evenly across all lobules. Scale bar indicates 1000 μm.
The other main cell types that reside in the cerebellum are granule cells and GABAergic interneurons derived from the EGL and VZ respectively. The EGL has been extensively characterised and is virtually entirely composed of GCPs that emanate from the rhombic lip. Their development sees their migration from the upper proliferative zone of the EGL to the lower differentiation zone, and then downward past the PCL to their terminal position in the IGL. As well as the inward migration of GCPs, there is an “inside-out” migration of cerebellar GABA-ergic interneurons from the ventricular zone. All GABA-ergic interneurons go through a similar differentiation sequence which involves early expression of the essential transcription factor Ptf1a for GABA-ergic specification, and a post-mitotic Pax-2 positive intermediate which is known to linger in the PWM for one to several days before reaching its final location (Leto, Bartolini and Rossi, 2010). These progenitors develop sequentially, beginning with the interneurons of the DCN followed by Golgi and Lugaro cells of the GL in the embryonic and early postnatal period (Leto et al., 2016). The final interneurons to mature are the basket and stellate cells of the ML which are derived postnatally from a pool of Pax-2 progenitors in the PWM.

Within the EGL, pPERK and pIRE1-positive cells predominantly resided within the upper proliferative zone. The expansion of GCPs in the EGL is driven by Sonic Hedgehog (Shh) expressed by PCs (Wechsler-Reya and Scott, 1999). Extrinsic factors such as vitronectin in the lower EGL impede Shh signalling and induce GCP maturation. Indeed dysregulation of Shh signalling is frequently used to model medulloblastoma. Such a model was serendipitously uncovered by the Popko group during an investigation of the effects of interferon-γ on the CNS (Corbin et al., 1996). Later it was uncovered that IFNγ had induced increased GCP expansion via increased activation and dysregulation of the Shh signalling pathway in a STAT1-dependent manner. Using a tetracycline-controllable system, models of cerebellar dysplasia or medulloblastoma were induced by ectopic expression of IFNγ in astrocytes (Lin et al., 2004; Wang et al., 2004). In a further investigation of this tunable system Lin et al., reported that deregulated PERK signalling promoted IFNγ-induced medulloblastoma formation (Lin et al., 2011). Of significance is their observation of pPERK- positive cells in the expanded GCP population (Fig. 3.25 B and C).
This supports our finding of pPERK expression in proliferating GCPs in the EGL. Unfortunately, in this study tissue was analysed only at 21 days, a timepoint at which the EGL has disappeared under normal conditions, and therefore never compared this deregulated expansion of GCPs with that of normal GCP development. Indeed the presence of PERK activation is consistently described in pathological terms in this study whereas our observations would suggest that it is part of normal GCP development. In agreement with this chapter’s findings, they report an absence of pPERK staining in other cells of the WT cerebellum. Similarly, they did not detect later downstream effectors of PERK signalling and reported no expression of ATF4 in GCPs. They did observe pεIF2α staining in medulloblastoma tissue although this did not appear to correspond with pPERK staining pattern (Lin et al., 2011).

The EGL gradually subsides during cortical maturation in the second and third postnatal week and we observed a simultaneous decrease in density of pPERK-positive cells between P7 and P17 in the EGL, IGL and PWM (p < 0.01, p < 0.01, p < 0.05 respectively). Additionally both cells in the EGL and PWM appear to be highly-proliferative at early timepoints and both recede during the same time period. Recently it has been shown that PC-derived Shh simultaneously drives a proliferative compartment of NSCs in the PWM as well as the expansion of GCPs in the EGL (Fleming et al., 2013a).

The germinal niche of the cerebellar PWM is less well characterised but several independent groups have greatly advanced our understanding in recent years. The proliferating progenitors of the postnatal PWM are known to generate cerebellar interneurons and astrocytes (Zhang and Goldman, 1996; Silbereis et al., 2009). In an important study by Lee et al., GCPs were FACS sorted from P7 cerebellum. Of the
remaining 10% of cerebellar cells, one third expressed neural stem cell markers such as CD133, also known as prominin-1. When CD133+ cells were depleted of lineage-specified cells (using O4 for oligodendrocytes, TAPA-1 for astrocytes and PSA-NCAM for neurons), the remaining unspecified cells constituted 1-3% of the original non-GCP cohort at P7. Detection of CD133 in P7 cerebellar tissue revealed positive cells in lobular PWM and occasionally the IGL but was absent from the ML and EGL. They went on to show that the majority of these CD133+ lineage-negative cells became parvalbumin-positive neurons of the ML, i.e. basket cells and stellate cells (Lee et al. 2005). The spatial distribution of CD133 is strikingly similar to that observed for pPERK at the same timepoint in this study. The low percentage of CD133 cells (1-3%) compared to all non-GCPs in the cerebellum corresponds quite closely to our quantification of pPERK expression in ~1% of all PWM cells at P7 in Chapter 4 (Fig. 4.5). Jászai et al., also reported a similar distribution pattern for CD133 cells to what is observed for pPERK in the PWM and IGL in P10 cerebellum and noted its absence in the ML and EGL (Jászai et al., 2013).

In an extensive investigation using genetically induced fate mapping analyses, CD133+, tenascin C\textsuperscript{low} cells were found to give rise to ML interneurons via generation of Ptf1a+ intermediates as well as cerebellar astrocytes via CD15+ precursors. Furthermore, this bipotential neural progenitor proliferated in response to PC-derived Shh (Fleming et al., 2013b). Independent groups have found evidence to support this role of a bipotential progenitor in the PWM as the sole source of cerebellar interneurons which proliferates in response to Shh (De Luca et al., 2015; Parmigiani et al., 2015).

Although much remains to be learned about CD133 progenitors in the cerebellum, it would appear that pPERK positive cells share many overlapping characteristics: similar cell density at P7 and P10 (other ages were not reported in the literature), relative distribution in PWM and IGL, regular occurrence in all lobules, active early proliferation status and apparent outward trajectory from the DCN to the periphery. Although a full developmental profile for CD133 is absent, the gradual decrease and loss of pPERK cells is within the expected timeframe for CD133 according to the known maturation program of cerebellar interneurons.
It would be interesting to know whether increased Shh signalling in the IFNγ-induced medulloblastoma model could similarly increase proliferation of pPERK positive cells in the PWM as it had done in the EGL. In the dosing regimen used, levels of IFNγ were first detectable at day 10, a time at which virtually all cerebellar interneurons have already been generated and progenitors may no longer be responsive to Shh-induced proliferation so this might not have been examined in this model (Weisheit et al., 2006; Fleming et al., 2013b). PERK signalling is tightly regulated in a feedback loop via ATF4-mediated induction of Growth arrest and DNA damage 34 (GADD34), which dephosphorylates eIF2α. Lin et al., employed an inactive GADD34 mutant mouse to model deregulated PERK signalling in medulloblastoma formation. In addition to the tumours observed in the EGL and ML in WT mice, tumour cells were also present in the white matter and IGL of GADD34 mutant mice. Interestingly this effect was not mediated by increased sensitisation of the Shh pathway as Gli1 and Shh mRNA levels were unchanged (Lin et al., 2011). It was suggested that deregulated PERK signalling increased the invasiveness of hyperplastic GCPs but perhaps the possibility that these cells were derived from Shh-responsive PWM progenitors should not be ruled out.

In summary, a comprehensive profile of UPR signalling in the normally developing cerebellar cortex has been undertaken, incorporating all three initiation pathways and classical downstream targets. Despite reports implicating the UPR in patterned PC neurodegeneration, parasagittal PC topography did not influence expression of UPR proteins in the normal cerebellum. Analysis of UPR gene expression showed that ATF6 and XBP1 mRNA increased significantly in the cerebellum with age but no changes were observed in relative levels of GRP78, GRP94 or PDI. Immunohistochemical analysis revealed time-dependent and cell-specific changes in UPR signalling in the cerebellar cortex. Activation of PERK and IRE1 signalling was observed for the first time in normally developing GCPs. A second pPERK-positive population was observed in the PWM and IGL in the developing cerebellum which also has not been described before. These populations may be relevant to studies of medulloblastoma and warrant further investigation to determine what cell-types are expressing them.
CHAPTER 4

PROFILE OF THE UPR DURING IN VIVO MYELINATION

The majority of this chapter has been published in the following papers:


Myelination in the human brain is said not to be complete until the third decade of life (Baumann and Pham-Dinh, 2001). It is increasingly recognised as a plastic process however which can adjust node number, length and thickness in response to neuronal activity, learning, social changes and even gut bacteria, as well as disease (Bengtsson et al., 2005; Scholz et al., 2009; Hu et al., 2011; Liu et al., 2012; Makinodan et al., 2012; Gibson et al., 2014; McKenzie et al., 2014; Hoban et al., 2016). Nonetheless the principal phase for myelination occurs in an intense period after birth in both human and rat CNS (Barateiro and Fernandes, 2014)(Fig. 4.1).

Myelination proceeds out from the brainstem extending through the cerebellum and cerebrum in a central to peripheral direction so that the vast majority of brain regions are myelinated within the first 3 weeks in rat and first 12 months in humans (Van der
Knaap and Valk, 1989; Hamano et al., 1998; Baumann and Pham-Dinh, 2001; Aubert-Broche et al., 2008; Downes and Mullins, 2014) (Fig. 4.1). In both species, myelination occurs through a similar program of cellular differentiation (Craig et al., 2003; Windrem et al., 2004; Dean et al., 2011; Barateiro and Fernandes, 2014). Many of the developmental processes of OPC recruitment, migration, process extension, wrapping and myelin compaction are similarly replicated by adult OPCs during repair of denuded axons in MS lesions but the mechanisms that underlie these processes are still to be fully understood.

It is estimated that an oligodendrocyte increases its surface area at a rate of 5-50 x 10^3 µm²/cell/day during active myelination in the postnatal rat (Baron and Hoekstra, 2010). Myelin components are synthesised, transported and inserted into the oligodendrocyte membrane in a regulated manner (Larocca and Rodriguez-Gabin, 2002). The principal site of synthesis for membranous proteins and lipids is the endoplasmic reticulum (ER). The smooth ER, particularly the endomembrane component, is responsible for membrane lipid biogenesis including the synthesis of phospholipids and galactosylceramide, the precursor to myelin lipids galactocerebrosides (GalC) and sulfatide (Fagone and Jackowski, 2009; Blom, Somerharju and Ikonen, 2011). For most myelin proteins (with the exception of MBP), ribosomes studded on the rough ER direct simultaneous translation and translocation into the ER lumen. Many myelin proteins, such as proteolipid protein (PLP), utilise ‘lipid rafts’ enriched in cholesterol and glycosphingolipids for their correct transport and insertion into myelin membrane (Jackman, Ishii and Bansal, 2009). The simultaneous ensheathment of multiple axons by a single oligodendrocyte occurs in a short time-window, after which the cell loses capacity to myelinate (5 hours in zebrafish, 12-18 hours in the rat) (Watkins et al., 2008; Czopka, Ffrench-Constant and Lyons, 2013). The metabolic requirements of a myelinating oligodendrocyte are therefore extremely high (Bradl and Lassmann, 2010). The estimated volume of membrane to be synthesised weighs 100 times that of its cell body therefore the expected burden on the endoplasmic reticulum (ER) of myelinating oligodendrocytes is considerable. This leads to the hypothesis that the UPR is activated in differentiating oligodendrocytes due to a necessity for increased ER capacity.
4.2 RATIONALE AND HYPOTHESIS

The dramatic shift from a motile precursor to a specialist membrane-synthesising cell applies to both young and adult OPCs during differentiation. In this respect, the study of developmental myelination is biologically and physiologically relevant to myelinating oligodendrocytes after injury. The developing rat cerebellum provides a well-characterised model in which myelination proceeds in a highly-regulated temporal and spatial sequence. Its laminar structure has allowed identification of prospective white matter tracts before they are myelinated, facilitating in-depth analyses of the morphology and immunohistochemical characteristics of oligodendrocytes during their maturation (Ghandour et al., 1980; Hartman et al., 1982; Monge et al., 1986; Ghandour and Skoff, 1988; Reynolds and Wilkin, 1988; Friedman et al., 1989; Warrington and Pfeiffer, 1992; Levine, Stinson and Lee, 1993; Coffey and McDermott, 1997; Li, Hertzberg and Nagy, 1997). The cerebellum is therefore ideal for categorising distinct stages of myelination and for the study of respective changes in UPR-associated molecules.

Hypothesis:

The UPR is activated during myelination of white matter tracts in vivo.

4.3 AIMS

- classify distinct stages of myelination with respect to specific tracts in order to identify critical time points for examination of the UPR in oligodendrocytes in maturing cerebellum.
- report changes in glial cell populations in developing white matter tracts.
- quantify changes in the activation of UPR initiators with respect to myelin progression.
- quantify changes in downstream targets of the UPR.
- confirm identity of cells positive for UPR markers.
4.4 RESULTS

4.4.1 Milestones in the myelination of cerebellar tracts III and IV

Stages of myelination in cerebellar lobes III and IV were qualitatively assessed using immunohistochemistry against early (DM20/PLP) and late (MOG) myelin markers and oligodendrocyte-lineage cells were identified using olig2 antibody (Fig. 4.2). The DM20/PLP antibody detects newly-differentiating oligodendrocytes making initial contact with axons as well as mature myelin. DM20 and PLP are myelin-specific protein isoforms encoded by the PLP gene. They are members of the highly conserved lipophilin family of transmembrane proteins that are also represented in Drosophila and silkworms (Stecca et al., 2000). DM20 is a smaller gene splice variant of PLP expressed on the surface of multi-branched, NG2-negative oligodendrocytes as they contact axons, representing the interim state between NG2-positive OPCs and mature myelinating oligodendrocytes (Trapp et al., 1997; Kukley, Nishiyama and Dietrich, 2010). PLP is a major structural protein, specific to CNS myelin, that appears to have arisen in amphibians and is synthesised by oligodendrocytes only after ensheathment has begun (Hartman, Agrawal, Agrawal, & Kalmbach, 1982). Myelin oligodendrocyte protein (MOG) is a highly-conserved, mammalian-specific myelin constituent and one of the last myelin-specific proteins to be expressed. It is considered a surface marker of fully differentiated oligodendrocytes (Baumann and Pham-Dinh, 2001).

The absence of both DM20/PLP and MOG immunoreactivity in lobes III and IV was used to define tissue “pre-myelination”. This was observed in prospective white matter tracts of lobes III and IV at P7 (Fig. 4.2). Moderate staining for DM20/PLP was observed alongside faint MOG staining at P10, indicating that the differentiation of OPCs and the initial active phase of myelination had been initiated. The area and intensity of staining of both proteins increased at P14 and P17 signifying accelerated myelination within the rapidly expanding WM tracts. Myelination was deemed to be complete in adult tissue, defined as age $\geq$ 6 months. Immunoreactivity of WM tracts was equally intense for DM20/PLP and MOG antibodies. This time point represented a stage in which mature oligodendrocytes were “post-myelination” and in a phase of myelin maintenance.
Figure 4.2. Myelination of cerebellar tracts in postnatal rat cerebellum. Olig2 is a marker for the oligodendrocyte lineage (LH panels). PLP is present in compacted internodal myelin, whereas its splice variant, DM20, is expressed on the cell-surface and processes of immature myelinating cells as they begin to contact axons (middle panels). MOG, a late-stage myelin marker, is typically expressed 1-2 days after PLP (RH panels). Primary antibody staining was visualised by DAB immunohistochemistry and nuclei were counterstained with haematoxylin. Scale bar = 50µm.
To further characterise the myelination program of the cerebellum total RNA was extracted at each timepoint and analysed by qPCR for myelin-specific genes. PLP, MBP and MOG mRNA levels showed similar patterns of upregulation, increasing dramatically from P7 to P17 and maintained at an elevated though slightly lower in adult tissue (Fig. 4.3).

Figure 4.3. Profile of myelin protein gene expression in developing cerebellum (n=3). PLP, MBP and MOG gene expression (top, middle and bottom respectively) exhibited similar expression profiles which increased from P7 to a maximum at P17 and remained at lower but elevated levels in adulthood.
4.4.2 Glial cell population changes during myelination

Cell density of prospective white matter tracts in lobes III and IV fluctuated significantly over time (Fig. 4.4A). Total cell density increased by 30% from P7 to P10 (7,744±743 and 10,110±508 cells per mm²). Thereafter, it reduced two-fold at P14 and ten-fold in adult (4,495±403 and 978±153 cells per mm²) (Fig. 4.4A, LH panel). The density of oligodendrocyte lineage cells, as identified by expression of the transcription factor olig2, increased significantly from P7 to maximal density at P14 (1,243±110 and 2,885±76 cells per mm²) after which it decreased to significantly lower levels (692±31 cells per mm² in adult) (Fig. 4.4A, middle panel). The proportion of oligodendroglial cells in developing tracts, when compared to total cell number, was relatively constant at P7 and P10 (20.7±1.8%, and 21.5±2.3%), increased significantly to 87.2±4.8% at P17 and stabilised at 70.8±3.2% in adult tissue (Fig. 4.4A, RH panel). Likewise, population changes in other glial cell types were observed over time. As reported previously, microglia undergo dramatic morphological changes during development (Ashwell, 1990). Prior to myelination, prospective tracts contained amoeboid microglial cells with thick short filopodia at P7 and P10, which gave way to smaller cell bodies with ramified branches, characteristic of “resting” microglia in older animals (Fig. 4.5 and insets). The area of Iba1 staining almost tripled between P7 and P14 (11.0±0.6% to 29.7±3.4%) and decreased significantly in adult tissue (2.7±0.6%) (Fig. 4.4B, LH panel). GFAP is a well-characterised marker of terminally differentiated astrocytes (Molofsky et al., 2012). GFAP antibody stained cells weakly at P7 and increasingly delineated the morphology of maturing cells, revealing thickened processes and darkly stained cell bodies at P14 and the characteristic fine branching of fibrous astrocytes at adulthood (Fig. 4.5 and inserts). The area comprised of GFAP-positive cells more than doubled between P7 and P14 (25.1±1.8% and 58.3±8.3%) and decreased in adult tracts (13.7±1.4%). This decrease was significant between P10 and adult (33.3±1.9% vs 13.7±1.4%) and between P14 and adult (58.3±8.3% vs 13.7±1.4%, Fig. 4.4B, RH panel).
Figure 4.4. Alterations in cell density during myelination. (A) Cell density of developing tracts changed significantly over time, reaching a maximum density in lobules III and IV at P10, and a relative 10-fold decrease in adult tissue when compared to the peak cell density. Olig2-positive cells reached a maximum density at P14, significantly higher than levels at P7 or adult. When normalised to the average cell density of each time point, the proportion of olig2-positive cells increased over time, reaching a significant peak at P17 vs. P7 and P10, and remained elevated in adult white matter tissue compared to early time points. (B) There was a significant rise in the percentage area positive for the microglial marker Iba1 at P14 vs. adult tissue. A significant increase in percentage area of GFAP expression was observed between P10 and P14 vs. adult. Statistical analyses were carried out using Kruskal-Wallis tests and Dunn’s post-tests. Data are mean±SEM (n=3). * p<0.05, ** p<0.01, *** p<0.001.
Figure 4.5. Glial cell population changes in the developing cerebellum. Antibodies against GFAP and Iba1 were used to detect astrocytes and microglia respectively. Scale bar = 20µm.

4.4.3 **ER stress sensors are differentially activated in white matter tracts undergoing myelination**

Subsequent to determination of myelination staging and quantification of glial cell population changes in developing tracts, the activation status of UPR initiators was
analysed to determine whether UPR signalling was present during developmental myelination.

Antibodies specific for the phosphorylated (activated) form of PERK detected isolated cells at P7, P10 and P14, representing ≤1% of the total cell population of young white matter tracts (Fig. 4.6). In adult tissue, less intensely stained pPERK-positive ramified cells were observed. Although the increase in pPERK-positive cells was significant (9.2±2.5% vs 0.5±0.3% at P14, Fig. 4.6), downstream indicators of PERK activation, peIF2α and CHOP, were not detectable at any age (Fig. 3.19). The unique patterning of pPERK in the developing cerebellar cortex combined with the temporal and spatial expression observed in the white matter indicated that distinct cell populations were represented at different stages, as explored in Chapter 3.

![Figure 4.6. pPERK expression in developing rat cerebellar tracts. Isolated, strongly-stained cells were present at low numbers at postnatal ages. Less intensely-stained process-bearing cells were observed at P17 and adult. Scale bar = 20µm. A significant increase in pPERK-positive cells was present in adult tissue. Statistical analysis was carried out using Kruskal-Wallis test and Dunn’s post-test. Data are mean±SEM (n=3 for all but P7, n=5). ** p<0.01.](image)

In contrast, the percentage of phosphorylated (activated) IRE1-positive cells increased significantly to widespread levels at P14 and P17 compared to white
matter tracts before myelination (P7) (64.0±13.6% vs 45.0±7.6%, Fig. 4.7). It returned to significantly lower levels in myelinated adult tissue (Fig. 4.7). This antibody has been used on human and mouse paraffin-processed tissue investigating the UPR in tau pathology in which it is associated with granulovacuolar lesions (Hoozemans et al., 2009; Nijholt et al., 2012; Köhler, Dinekov and Götz, 2014). In this study, detection of pIRE1 varied from a punctate or granular appearance to a more diffusely-localised cellular staining pattern which was also observed by Nijholt et al. (2012). In our hands, pIRE1 immunoreactivity lightly stained adult white matter tracts and this effect was also reported by Kohler et al. when using this antibody in normal adult mouse (2014).

Figure 4.7. pIRE1 expression in developing white matter tracts. pIRE1 expression was most notable in actively myelinating tracts compared to time points pre- and post-myelination. Scale bar = 20µm. The percentage of pIRE1-positive cells significantly increased from P7 to over 40% of cells at P14 and P17 and returned to low levels in adult tissue. Statistical analysis was carried out using Kruskal-Wallis test and Dunn’s post-test. Data are mean±SEM (n=3 for P7 and adult; n=4 for P10 and P14; n=5 for P17). * p<0.05, ** p<0.01

Cells positive for ATF6 were present in white matter tracts at all time points studied (Fig. 4.8A). There was a significant increase in the percentage of ATF6-positive cells between P17 and adult (11.2±2.9% vs 46.2±5.1%, Fig. 4.8B LH panel). ATF6
is a transmembrane protein on the ER lumen when inactive. During ER stress, it is proteolytically cleaved and the active transcription factor translocates to the nucleus. Here, nuclear-localised ATF6 (ATF6(N)) was observed only at P7, P10 and P14, but was entirely absent in older animals. A concurrent increase in non-nuclear ATF6-positive cells (ATF6(C)) was observed at these later time points (insets, Fig. 4.8A). This shift between nuclear and non-nuclear staining in the population of ATF6-positive cells was significant between P10 and P17 (68.3±11.4% vs 0.0±0.0% for ATF6(N)), and between P10 and adult (68.3±11.4% vs 0.0±0.0% for ATF6(N)) (Fig. 4.8B, middle and RH panel). Significant differences in the percentage of ATF6(N)-stained cells within the total cell population in white matter were observed between P10 and P17 (19.1±5.6% and 0.0±0.0%) and between P10 and adult (19.1±5.6% and 0.0±0.0%) (Fig. 4.8B, lower LH panel). Similarly, the increase in the percentage of ATF6(C)-positive cells in the total cell population was significant between P10 and adult (7.7±2.3% vs 46.2±5.1%), and between P14 and adult (12.0±5.7% vs 46.2±5.1%, Fig. 4.8B, lower RH panel). As noted in the previous chapter, ATF6-positive cells displayed the typical distribution and morphology of oligodendrocytes in adult tissue (Fig. 3.10). In addition, dual-chromogenic IHC using DAB and liquid permanent red suggested ATF6 expression in PWM olig2+ cells at P10 (Appendix, Fig. A4.1).
Figure 4.8. ATF6 expression in developing rat cerebellar tracts. (A) During myelination, ATF6 positive cells were primarily nuclear, indicative of the active transcription factor. Post-myelination, no ATF6-positive cells exhibited nuclear localisation. Positively-stained cells showed nuclear localisation at younger ages (P7, P10, P14) and was replaced by non-nuclear staining at later time points (see insets). Scale bar = 20µm. (B) The total number of cells positive for ATF6 increased significantly between P17 and adult. From this population of ATF6-positive cells, the percentage with nuclear staining was significantly higher at P10 vs. P17 and adult. Similarly, the percentage of non-nuclear stained cells was significantly raised.
at P17 and adult vs. P10. The percentage of cells with nuclear-localised ATF6 was also significantly raised within the total cell population at P10 vs. P17 and adult. A significant rise in the percentage of non-nuclear ATF6 stained cells in the total cell population was observed between P10 and P14 vs. adult tissue. Statistical analyses were carried out using Kruskal-Wallis tests and Dunn’s post-tests. Data are mean±SEM. (n=3) * p<0.05, ** p<0.01, *** p<0.001.

4.4.4 UPR targets are significantly upregulated and remain elevated after myelination

As the density of oligodendrocytes increased (Fig. 4.4), we observed increasingly abundant numbers of cells positive for typically upregulated targets of the UPR. The majority of these displayed the typical morphology of mature oligodendrocytes (Fig. 4.9).

A sustained increase in the density of GRP78-positive cells was observed over time and this was significantly different at P7 and P10 compared to P17 and adult (5.7±2.4% and 10.8±2.1% vs 43.4±5.3% and 85.8±14.4%) (Fig. 4.10, Fig. 4.9 LH panel). The number of cells positive for GRP94 was significantly different between P7 and all other time points (P7 15.5±1.3% vs P10 32.7±2.6%, P14 31.9±3.6%, P17 55.8±5.8% and adult 46.5±8.4%) (Fig. 4.10, Fig. 4.9 middle panel). A significant increase in cells positive for PDI was also observed between P7 and P10 versus P17 and adult (1.9±0.5% and 2.3±0.3% vs 21.2±2.9% and 31.2±4.7%) (Fig. 4.10, Fig. 4.9 RH panel). Detectable calreticulin expression, however, was not observed within white matter tracts in this study.
Figure 4.9. Expression of GRP78, GRP94 and PDI in developing rat cerebellar tracts. The number of positive cells for these downstream targets increased after the activation of UPR transducers and remained elevated in adult tissue. Primary antibody staining was visualised by DAB immunohistochemistry and nuclei were counterstained with haematoxylin. Scale bar = 20µm
Figure 4.10. Quantification of GRP78-, GRP94- and PDI-positive cells in developing rat cerebellar tracts. The ratio of GRP78 positive cells increased significantly between P7 and P10 vs. P17 and adult. Cells positive for the ER chaperone GRP94 increased significantly between P7 vs. P10 and P14 and increased further at P17 and adult. Cells positive for the folding enzyme PDI were significant at P17 and adult vs. P7 and P10. Statistical analyses were carried out using Kruskal-Wallis tests and Dunn’s post-tests. Data are mean±SEM GRP78: n=4 for P7, P14 and adult; n=5 for P10 and P17. GRP94: n=5 for P7; n=4 for P10; n=6 for P14; n=3 for P17 and adult. PDI: n=3 for all but P17 when n=4. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).

4.4.5 UPR signalling pathways are differentially activated prior to and during myelination

To assess the relative contributions of each arm of the UPR at each time point a 2-Way ANOVA and Bonferroni post hoc test was used to determine significant differences in the number of pPERK, pIRE1 and ATF6(N)-positive cells over time.
(Fig. 4.11A). A significant interaction was found, \( p<0.0001 \), and the effect of time points and of UPR initiators each significantly influenced this, \( p<0.0001 \) for each.

Before the onset of myelination at P7, nuclear-localised ATF6 was observed in twice as many cells as activated IRE1 \((697.0\pm268 \text{ vs } 361.6\pm218 \text{ cells per mm}^2)\), which was more abundant again than pPERK \((104\pm20.3 \text{ cells per mm}^2)\). During the early active phase of myelination at P10, both ATF6(N) and pIRE1-positive cells increased to \( \sim2,000 \) cells per mm\(^2\), significantly higher than pPERK which was unchanged \( (p<0.001) \). At P14, there was a 10-fold difference between the numbers of pIRE1 and ATF6(N)-positive cells \((2875.6\pm612.5 \text{ vs } 281.9\pm153.2 \text{ cells per mm}^2, p<0.001)\). Although ATF6(N) was no longer detected after P14, pIRE1-positive cells declined at a slower rate. At P17, significant differences persisted between pIRE1 and ATF6(N) \((1183.7\pm198.7 \text{ vs } 0.0\pm0.0 \text{ cells per mm}^2, p<0.01)\) and between pIRE1 and pPERK \((71.40.0\pm53.3 \text{ cells per mm}^2, p<0.05)\). In post-myelinated adult tissue there were no longer any significant positive-cell population differences between active UPR initiators (Table 4.1).

When statistical tests were applied to detect variation in UPR targets at these time points, a significant difference was also found and both time point and UPR initiator each significantly influenced this (Fig. 4.11B, \( p<0.0001 \) for all). Prior to myelination at P7, the number of positive cells for GRP94 was significantly greater than for GRP78 or PDI \((1202.4\pm97.4 \text{ vs } 443.1\pm187.3 \text{ and } 146.8\pm37.2 \text{ cells per mm}^2, p<0.01 \text{ and } p<0.001 \text{ respectively})\). At P10 these differences were more dramatic and GRP94-positive cells were 3-fold higher than for GRP78 and 14-fold higher than for PDI \((3303.4\pm262.6 \text{ vs } 1087.6\pm212 \text{ and } 232.8\pm32.5 \text{ cells per mm}^2, \text{ both } p<0.001\), despite GRP78-positive cells having doubled and also being significantly different to PDI \( (p<0.01) \). PDI-positive cells did not increase substantially until P14 \((952.0\pm132.5 \text{ cells per mm}^2)\). No significant differences were detected between the UPR targets at P14, P17 or adult (Table 4.1).
Figure 4.11. Temporal expression of UPR proteins. (A) UPR transducers. Key: * denotes significant difference between pIRE1 and pPERK, \( p < 0.001 \). ● denotes significant difference between pIRE1 and pPERK, \( p < 0.05 \). # denotes significant difference between pIRE1 and ATF6(N), \( p < 0.001 \). ^ denotes significant difference between pIRE1 and ATF6(N), \( p < 0.01 \). ♦ denotes significant difference between ATF6(N) and pPERK, \( p < 0.001 \). (B) Downstream target molecules of the UPR. # denotes significant difference between GRP94 and PDI \( p < 0.001 \), ● denotes significant difference between GRP78 and PDI \( p < 0.01 \). * and ♦ denote significant differences between GRP78 and GRP94, \( p < 0.01 \) and \( p < 0.001 \) respectively. Statistical analyses were carried out using Two-Way ANOVA and Bonferroni post-tests.
Table 4.1. Expression profile of UPR-associated molecules in developing white matter tracts.

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Key: - = not detected; + = 1-19%; ++ = 20-39%; +++ = 40-59%; ++++ = 60-79%; +++++ = 80-100% of cells.

4.4.6 *ATF6(N) and its downstream targets are closely associated with oligodendrocytes*

Cerebellar tracts at P14 represent a time of flux in which oligodendrocytes, astrocytes and microglia can be observed in a mixture of polarised states i.e., OPC vs mature oligodendrocyte and “activated” vs “resting” morphologies associated with astrocytes and microglia (Fig. 4.4 and Fig. 4.5). To determine which populations possessed an activated UPR, dual-immunofluorescent staining was performed with cell-specific markers. Although chromogenic staining demonstrated strong pIRE-1 expression in white matter tract cells, this antibody had not been validated for use on unfixed frozen tissue and attempts to dual-label using this antibody were unsuccessful.

Staining for ATF6 expression was localised both inside and outside the nucleus of olig2-positive cells (Fig. 4.12, LH panel). Non-nuclear ATF6 expression was also occasionally associated with microglia, but absent in astrocytes (Fig. 4.12 middle and RH panels).
Figure 4.12. ATF6 is activated in oligodendrocytes during myelination. ATF6 (red) is shown with cell-specific markers (green) and DAPI nuclear staining (blue) in white matter tracts of P14 rat cerebellum. Both nuclear and non-nuclear localised ATF6 expression is observed in olig2-positive cells. ATF6 is observed outside the nucleus in microglial cells indicated by OX-42. ATF6 was not associated with astrocytes revealed by GFAP staining. Arrows indicate ATF6 expression outside the nucleus (inactive) in oligodendrocytes and microglia. Arrowheads show nuclear staining indicative of cleaved ATF6 transcription factor in oligodendrocytes. Scale bar = 5µm.
4.4.7 Downstream targets of ATF6 are highly expressed in oligodendrocytes

As GRP78, GRP94 and PDI are all strongly induced by ATF6(N), and to a lesser extent by spliced XBP1 (Shoulders et al., 2013), it was next assessed whether these ER folding proteins would be specifically up-regulated in oligodendrocytes in a period of active myelination. At P14, GRP78, GRP94 and PDI were almost exclusively associated with olig2-positive cells (Fig. 4.13, top row). Although the presence of occasional positive cells that were olig2-negative was noted, GRP78, GRP94 and PDI did not appear to co-localise with GFAP or OX-42 staining (for astrocytes or microglia, respectively) (Fig. 4.13, middle and bottom rows). It is possible that GFAP-negative astrocytes or OX42-low positive microglia could account for these cells. In addition, a minority of olig2-positive cells did not express GRP78, GRP94 or PDI at P14. The high expression of these molecules in mature tracts may indicate that this negative population may represent immature oligodendrocyte precursors. The minority of olig2-positive cells did not express GRP78, GRP94 or PDI at P14 may represent immature oligodendrocyte precursors, given the high expression of these molecules in adult tracts populated by mature oligodendrocytes.
Figure 4.13. UPR target proteins strongly associate with oligodendrocyte cells. GRP78 (LH panels), GRP94 (middle panels) and PDI (RH panels) are labelled red and cell-specific markers green, with DAPI nuclear staining in blue, in white matter tracts of P14 rat cerebellum. GRP78 is expressed in olig2-positive cells but does not associate with microglia or astrocytes as revealed by OX-42 and GFAP staining, respectively. Similarly, GRP94 and PDI strongly associate with oligodendrocytes and are absent in microglia and astrocytes. Scale bar = 5µm
4.5 DISCUSSION

Although the rat cerebellum is populated by oligodendrocyte precursors before birth, myelination is initiated only at P10, as cerebellar cortical neurons mature ((Reynolds & Wilkin, 1988) and Fig. 4.2). Cell density decreases once myelination is initiated due to volumetric expansion, the exit of neuronal preursors, substantial astroglial death and the apoptosis of OPCs that fail to myelinate (Altman, 1982; Bandeira, Lent, & Herculano-Houzel, 2009; Krueger, Burne, & Raff, 1995). In this study, oligodendrocytes accounted for 20% of cells in prospective tracts at P10, rising to over 70% in adulthood. In keeping with other reports, microglial morphology was seen to change from an early amoeboid morphology (P7-P10), to a ramified shape consistent with a ‘resting’ phenotype (Ashwell, 1990; Salter and Beggs, 2014; Perez-pouchoulen, Vanryzin and Mccarthy, 2015). Similarly astrocytes became more finely branched as the cerebellum matured (Fig. 4.5).

It is estimated that a postnatal rat oligodendrocyte increases its surface area at a rate of $5-50 \times 10^3 \, \mu m^2/cell/day$ during active myelination (Baron & Hoekstra, 2010). As a site for the synthesis, processing and trafficking of membrane proteins and lipids, the ER is expected to face increased load during this process. This study defined the profile of expression of activated ER stress sensors ATF6, IRE1 and PERK and their downstream targets in cerebellar white matter tracts prior to myelination (P7), during axonal contact and initial myelin synthesis (P10), during accelerated myelin wrapping and maturation (P14 and P17) and at a post-myelination stage in fully mature tissue (adult).

Quantification of immunohistochemical staining revealed that PERK signalling was minimal during myelination, as confirmed by extremely low numbers of positive cells for p-PERK cells and the lack of detectable p-eIF2$\alpha$ or the transcription factor CHOP. It supports the findings of the Popko group, who have extensively investigated the role of PERK signalling as part of the integrated stress response (ISR) in oligodendrocytes (Lin et al., 2005, 2006, 2007, 2008, 2013; Hussien, Cavener and Popko, 2014; Hussien et al., 2015; Way et al., 2015; Clayton and Popko, 2016). Although normal developmental myelination has been described in oligodendrocyte-specific PERK-null mice, these mice were reported to have exacerbated disease in experimental autoimmune encephalomyelitis (EAE) (Hussien,
Cavener and Popko, 2014). Indeed, stimulation of PERK signalling was shown to significantly improve OL survival in EAE (Lin et al., 2013) and is now being investigated as a potentially therapeutic approach for multiple sclerosis (NCT02423083, 2000; Way et al., 2015). In this study, no expression of peIF2α or CHOP was observed at any time point. Similarly, no expression of peIF2α was observed in oligodendrocytes in normal adult spinal cord (Lin et al., 2013) and others also found CHOP staining to be undetectable in oligodendrocytes at P16 or adult mouse (Southwood et al., 2002, 2016; McLaughlin et al., 2007).

In addition, the relative number and spatial/temporal distributions of p-PERK-positive cells indicate that they do not belong to the oligodendrocyte-lineage and may represent a population of neuronal precursors engaged in peIF2α-independent modifications, potentially associated with autophagy (as discussed in chapter 3).

Interestingly, activation of ATF6 and IRE1 was detected in white matter tracts at P7, before the appearance of myelin proteins. ATF6 expression peaked at P10, while pIRE1 peaked at P14. The percentage of cells positive for pIRE1 was significantly greater at P14 and P17, when the rate of myelination is at its greatest, compared to time points pre- and post-myelination. Nuclear ATF6 was observed in cerebellar tracts at P7, P10 and P14, just prior to, and during, the initial phase of myelination, which precipitates a vast increase in membrane synthesis. ATF6 is known to increase phospholipid synthesis both independently of, and in conjunction with, XBP1 mRNA (Bommiasamy et al., 2009). Furthermore, ATF6 activation has been reported in response to membrane protein expression in the absence of stress (Maiuolo et al., 2011). To determine which cells possessed an activated UPR, dual-immunofluorescent staining was performed with cell-specific markers. In accordance with the chromogenic staining, nuclear and non-nuclear localisation of ATF6 was observed. It was most frequently associated with oligodendrocytes and occasionally in microglia. Unfortunately, immunofluorescent dual-labelling was unsuccessful with the p-IRE1 antibody. This antibody is validated by the manufacturer for paraffin IHC but not for immunofluorescence on frozen tissue and an absence of reports containing in vivo, brain cell-specific, fluorescent dual labelling with pIRE1 and XBP1 antibodies has been noted in general.
Although studies using the ATF6α−/− mouse show it to be developmentally-normal this may be due to compensatory functions by the ATF6β isoform as the double knockout (ATF6α−/− ATF6β−/−) is embryonically-lethal (Yamamoto et al., 2007). IRE1/XBP1 signalling in the brain has been shown to be beneficial in stress-resistance, aging and models of neurodegeneration (Mardones, Martinez and Hetz, 2015). The asynchronous activation of ATF6 and IRE1 signalling alters the pool of upregulated UPR targets, including those related to protein folding, trafficking and degradation, which may be a reflection of the shifting protein and lipid requirements of oligodendrocytes. The sequential expression of myelin proteins is well-characterised and is known to follow after initiation of lipid synthesis, particularly GalC and sulfatide, which also occurs at the ER (Ozgen et al., 2016). The lipid composition of myelin also changes during development, at first higher in phospholipids, similar to plasma membrane, and thereafter increasing in cholesterol and glycosphingolipids as it matures (Cuzner and Davison, 1968; Norton and Poduslo, 1973).

Changes in the levels of downstream targets of the UPR were also investigated. GRP78, GRP94 and PDI were all found to increase as myelination progressed and were significantly higher at P17 and adult vs. unmyelinated tissue at P7. The expression of all three proteins also remained elevated into adulthood. GRP78 has a broad range of protein clients and is capable of recognising unfolded nascent polypeptides of both glycosylated and non-glycosylated proteins (Otero, Lizak, & Hendershot, 2010). The more restricted protein clientele of GRP94 includes the LDL receptor family of proteins (Weekes et al., 2012), insulin growth factor 1 and integrins (Eletto, Dersh, & Argon, 2010), all of which have been shown to be selectively expressed by differentiating oligodendrocytes (From et al., 2014; Weekes et al., 2012; C. Zhao, Fancy, Franklin, & ffrench-Constant, 2009; S. Zhao et al., 2007).

The chaperone calreticulin (CRT) was not upregulated in myelinating tracts in our study. CRT acts as a major calcium buffer and as a partner in the calreticulin-calnexin cycle for the folding of glycoproteins in the ER. Despite being a target of the UPR, CRT is not required for MOG expression (Jung and Michalak, 2011) and its function as a calcium buffer can be compensated for by GRP94 (Argon and Simen, 1999) suggesting that it may be dispensable for myelination.
Although highest expression of GRP78, GRP94 and PDI occurred in mature tracts in the absence of UPR activation, high basal levels of such proteins are not uncommon. All three ER-resident proteins have been detected in myelin proteomic studies as well as cultured oligodendrocytes (Neri, Duchala and Macklin, 1997; Taylor et al., 2004; De Monasterio-Schrader et al., 2012). The high expression of non-nuclear ATF6 and ER chaperones in mature tracts was conspicuously associated with the cell morphology of mature oligodendrocytes (Fig. 4.8 and Fig. 4.9). ATF6(N) is known to have a short half-life (40 min - 3 hours) (Thuerauf et al., 2002; Teske et al., 2011). Its function as a strong inducer of all three chaperones (Adachi et al., 2008; Shoulders et al., 2013) and their high expression in mature white matter tracts suggests that low levels of ATF6(N) may still be transcriptionally active, although not detectable from histological staining. Dual staining for these molecules at P14 was strongly associated with oligodendrocytes, being seen rarely in microglia and not found in astrocytes. The minority of olig2-positive cells that did not express GRP78, GRP94 or PDI at P14 are thought to represent immature oligodendrocyte precursors.

These data suggest that oligodendrocytes may utilise a specialised UPR to cope with exceptional synthetic demand, such as that arising during myelinogenesis. It may be that, in this situation, oligodendrocytes are employing a strategy similar to that used by differentiating B lymphocytes, where the three arms of the UPR are differentially activated, with a notable suppression of the PERK pathway (Ma, Shimizu, Mann, Jin, & Hendershot, 2010). Since PERK signalling is known to result in a significant dampening of global protein synthesis, it is possible that a PERK-suppressing strategy would also appear beneficial in the case of active processes such as immunoglobulin production and myelination. CHOP is a downstream target of PERK signalling which is traditionally viewed as an effector of ER stress-induced apoptosis. Interestingly however, recent reports showed that selective CHOP overexpression in myelinating cells had no effect in development or adulthood under physiological or metabolic stress conditions (Southwood et al., 2016). This provides further evidence that a sophisticated and selective UPR has been adapted in oligodendrocytes.
In conclusion, quantification of immunohistochemical staining revealed that nuclear ATF6 and pIRE1 are significantly increased in an asynchronous fashion prior to, and during, myelination. In contrast, activation of PERK signalling was minimal, as confirmed by lack of detectable p-PERK, p-eIF2α or the transcription factor CHOP. Subsequent significant increases in the expression of GRP78, GRP94 and PDI were observed but this was not accompanied by a similar induction of CRT. This indicates a selective UPR is utilised by oligodendrocytes during myelination which omits PERK signalling and its associated block of protein translation and that UPR targets are continuously expressed during myelin maintenance.

Few studies compare induction of all three arms of the UPR simultaneously. This is important in understanding the potential role of the UPR in oligodendrocytes during demanding processes such as myelination of axons. Understanding which signalling pathways promote myelination could potentially help in the development of small-molecule drugs that can interfere with or augment these cascades, to ease cellular stress and provide a regenerative milieu in an otherwise pathological environment.
CHAPTER 5

THE EFFECTS ON OLIGODENDROCYTE DIFFERENTIATION INDUCED BY UPR MANIPULATION.
5.1 INTRODUCTION

The findings of the previous chapter demonstrated that a physiological UPR was active during the myelination phase of white matter tracts in \textit{in vivo} development. The next aim was to determine whether UPR signalling was specifically active, or indeed required, for the maturation of oligodendrocytes into myelinating cells. In the absence of transgenic models which could conditionally block UPR signalling under an oligodendrocyte-specific promoter such as CNPase or PLP, an \textit{in vitro} approach was applied using pharmacological inhibitors of UPR signalling on differentiating oligodendrocytes.

Various \textit{in vitro} models are available that faithfully reproduce the sequence and hallmarks of oligodendrocyte maturation and myelin formation (Madill \textit{et al.}, 2016). Primary OPCs can be isolated from CNS tissue, expanded and differentiated \textit{in vitro} with highly-predictable morphological and transcriptional changes. Immortalised oligodendrocyte cell lines have the added advantages of accelerated expansion and infinite growth whilst eliminating the need for fresh tissue. Mixed glial and co-culture systems facilitate axon-glia interactions and myelin wrapping. Organotypic brain slice cultures more closely mirror the complexity of \textit{in vivo} conditions as they support the interactions of all cellular players during myelination and retain the 3D cytoarchitecture of axonal tracts.

Until very recently, the best available pharmacological agents to modulate the UPR target the IRE1 and PERK signalling pathways with a noted absence of any specific compounds to target ATF6 (Hetz, Chevet and Harding, 2013; Maly and Papa, 2014). Guanabenz inhibits the de-phosphorylation of eIF2\(\alpha\), an important feedback loop of the PERK signalling pathway. The RNase activity of activated IRE1 can be directly blocked by salicylaldehyde-based compounds such as 4\(\mu\)8C, considered one of the best-in-class (Maly and Papa, 2014). Cell-based high-throughput screening approaches have now identified several compounds that can selectively modulate ATF6 signalling although these were not available at the time of this study (Gallagher \textit{et al.}, 2016a; Gallagher and Walter, 2016b; Plate \textit{et al.}, 2016). The lack of available compounds targeting ATF6 signalling previously led some studies to use general inhibitors of site-1 and/or site-2 proteases (Yamazaki \textit{et al.}, 2009; Nakajima \textit{et al.}, 2011; Liu \textit{et al.}, 2013; Bickler \textit{et al.}, 2015). These proteases are essential for
the cleavage and activation of ATF6 but are equally necessary for other substrates, in particular sterol regulatory-element binding proteins (SREBPs) (Ye et al., 2000; Okada et al., 2003). Like ATF6, SREBPs are ER luminal proteins which transit to the Golgi for the cleavage of an active transcription factor. They function as master regulators of lipid homeostasis and de novo lipid synthesis, including cholesterol (Eberlé et al., 2004; Ye and DeBose-Boyd, 2011). Given the established role of cholesterol synthesis not just as a rate-limiting step for membrane expansion but also as a necessity for myelin gene expression, it would not be feasible to employ such agents to investigate a role of ATF6 in oligodendrocyte differentiation (Saher, Quintes and Nave, 2011; Mathews et al., 2014). Therefore a gene silencing approach was undertaken to determine whether ATF6 signalling was required for oligodendrocyte differentiation.

5.2 RATIONALE AND HYPOTHESIS

As one of the most metabolically-demanding cells of the CNS, oligodendrocytes invest enormous energy on the coordination and control of axonal conduction via concentric membranes that are up to 100x the weight of the cell body (McTigue and Tripathi, 2008; Harris and Attwell, 2012). The UPR is a conserved response to increasing ER processing requirements which may be under stress in oligodendrocytes due to the volume of myelin lipid and protein synthesis and post-translational modifications which take place in the ER. Application of selective UPR inhibitors to in vitro culture systems could reveal whether UPR signalling systems are involved in oligodendrocyte maturation and which arms of this pathway are most significant.

Hypothesis

Modulation of UPR signalling pathways will impact oligodendrocyte differentiation.

5.3 AIMS

- To set up oligodendrocyte cell line and primary OPC culture models of oligodendrocyte maturation
- To screen selective agents acting on UPR pathways for effects on OPC maturation
- To test if such effects persist when applied in remyelinating organotypic slice cultures
5.4 RESULTS

5.4.1 Validation of O2A-myc cell line

To confirm that O2A-myc cells were representative of oligodendrocytes, cells were stained for the lineage marker olig2. All DAPI-stained nuclei of O2A-myc cultures were found to be positive for the olig2 transcription factor (Fig. 5.1).

Figure 5.1. O2A-myc cells express the olig2 transcription factor. All DAPI-stained nuclei colocalised with olig2 expression, shown at 20x (top row) and 40x (bottom row). Scale bar = 20 μm.

O2A-myc cells were cultured in OPC growth medium to assess whether they possessed features of OPCs. All O2A-myc cells cultured in OPC growth medium were positive for olig2 and NG2, a chondroitin sulphate proteoglycan expressed on the cell surface of OPCs (Fig. 5.2). NG2 expression delineated a bipolar and tripolar morphology in O2A-myc cells that is characteristic of immature OPCs.
Figure 5.2. O2A-myc cells cultured in OPC growth medium possess the phenotype of OPCs. All O2A-myc cells expressed the OPC marker NG2 and the olig2 transcription factor (left inset). NG2 expression outlined the characteristic bipolar and tripolar morphology characteristic of OPCs (right inset). Scale bar = 40μm (left) and 20μm (right).

To validate whether O2A-myc cells also replicate features of maturing oligodendrocytes, cells were cultured in the absence of growth factors in differentiation medium containing tri-iodothyronine and thyroxine (T3 and T4) hormones. When these cells were monitored by brightfield imaging, the increasing ramification of O2A-myc cell processes was noted (Fig. 5.3). After two days in differentiation medium multiple primary and secondary processes were observed. All cells were multi-branched by 3 days and there was an increase in the abundance of cells with tertiary processes. After 5 days in differentiation medium O2A-myc processes became exceedingly fine. Beyond this timepoint there was a decrease in adherent cells, sometimes leaving behind the fine lattice of processes on the cell culture substrate. This therefore became a cut-off point for differentiation studies.

Differentiating O2A-myc cells were also assessed for expression of markers for OPCs and mature oligodendrocytes. A multi-branched morphology and decreased NG2 expression was apparent after 2 days in differentiation medium (Fig 5.4). After 4 days NG2 antigenicity had been lost and cells were weakly positive for the mature myelin protein MBP.
Figure 5.3. O2A-myc cells become progressively ramified in differentiation medium. Cells initially exhibited bipolar and tripolar morphology characteristic of OPCs. After 2 days in differentiation medium more primary processes and secondary processes were observed. At 3 days all cells were multi-branched and there was increased tertiary branching. At 5 days O2A-myc processes were exceedingly fine. All images at 20x magnification.
Figure 5.4. O2A-myc cells cultured in differentiation medium attain features of maturing oligodendrocytes. After two days in differentiation medium fewer cells were positive for the immature marker NG2 (red, top row, 20x). O2A-myc cells attained multi-branched phenotype typical of maturing oligodendrocytes (bottom left, 40x). After 4 days in differentiation medium NG2 antigenicity had been lost (bottom right, 40x). Cells were weakly positive for the mature myelin marker MBP (green, bottom RH image). Scale bar = 20μm

5.4.2 UPR gene expression during oligodendrocyte differentiation

As O2A-myc cells exhibited classical features of the differentiation program of oligodendrocytes and with the advantage of being more easily expanded to large numbers, these cells were used to analyse changes in UPR gene expression during the OL differentiation program. O2A-myc cells were expanded in triplicate flasks and harvested for RNA after 0, 1, 3 and 5 days in differentiation medium. Samples were assayed by qPCR for myelin-specific genes PLP, MBP and MOG. All myelin genes were found to increase with time. The largest increase was observed for PLP which was >6-fold after 4 days. A 4-fold increase was observed for MBP, while MOG was the slowest to increase (>2 fold) (Fig. 5.5). This pattern follows the
classical order of the OL differentiation program \textit{in vivo} in which PLP is the first myelin protein expressed, followed by MBP, and lastly MOG 1-2 days later.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{fold_change.png}
\caption{O2A-myc cells cultured in differentiation medium upregulated myelin-specific genes. PLP, MBP and MOG mRNA levels all increased over time. Values are representative of three replicate flasks.}
\end{figure}

Activation of the UPR converges on the signalling mediators ATF6 and XBP1s for transcriptional induction of ER quality control proteins (Yamamoto \textit{et al.}, 2007; Teske \textit{et al.}, 2011). Gene expression levels for ATF6, XBP1u and XBP1s mRNA were analysed in O2A-myc cells cultured in differentiation medium at 0, 1, 3 and 5 days but no changes in mRNA levels of these genes were detected (Fig. 5.6).

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{ATF6_XBP1.png}
\caption{O2A-myc cells cultured in differentiation medium did not alter mRNA levels of ATF6, XBP1u or XBP1s. Values representative of three replicate flasks.}
\end{figure}
Gene expression levels of typical targets of the UPR; GRP78, GRP94 and PDI, were also quantified. Again, no changes in expression occurred in O2A-myc cells between 0 and 5 days in differentiation medium (Fig. 5.7).

![Figure 5.7](image)

**Figure 5.7.** O2A-myc cells cultured in differentiation medium did not alter mRNA levels of UPR targets. No changes were seen in expression levels of GRP78, GRP94 or PDI mRNA. Values representative of three replicate flasks.

5.4.3 Validation of primary OPC cultures

Although O2A-myc cells possess many characteristic features of early and maturing OPCs, they did not recapitulate all features of the oligodendrocyte differentiation program as they did not elaborate expansions of membrane or strongly express myelin proteins, critical processes in myelin formation. Primary OPCs can be isolated and differentiated *in vitro* and are typically used in the screening of potentially promyelinating compounds. As these cells could better reflect oligodendrocyte differentiation but are slower to grow and culture, an *in vitro* primary oligodendrocyte model was set up for the screening of effects on oligodendrocyte maturation by available compounds that modulate the UPR. Several methods of OPC preparation were investigated including isolation from the optic nerve, immunopanning and shake-off from mixed glia cultures. Highest yields were obtained using the shake-off method. In this method, mixed glia were cultured until a continuous monolayer formed on the bottom of the flask. Microglia were depleted in two steps and purified OPC cell suspensions were derived from mixed glia flasks shaken overnight at 37°C. OPCs were expanded in T25 flasks which could typically
take 1-2 weeks depending on yield. These cells were semi-adherent and were frequently found to spontaneously form oligospheres (Zhu et al., 2014).

Purity was assessed by expression of olig2, GFAP and DAPI staining in independent preparations of OPC cultures (Fig. 5.8, 5.9). DAPI-stained nuclei were over 95% positive for olig2 expression and this was consistent between flasks used for OPC expansion and between preparations derived from independent mixed glial cultures (Fig. 5.8). In consequence, the number of GFAP-positive contaminating cells was very low (Fig. 5.9).

Figure 5.8. Independent cultures of primary OPCs had consistently high purity. DAPI-stained nuclei were over 95% positive for the olig2 transcription factor in OPC cultures derived from separate expansion flasks and from separate isolations derived from independent mixed glia cultures. A minimum of three fields were quantified per well of OPCs derived from three individual expansion flasks per prep. Data presented with mean and SEM (n=3 wells).
Figure 5.9. Independent cultures of primary OPCs were consistently low in GFAP-positive contaminating cells. A representative image of OPC cultures stained for olig2 (red), GFAP (green) and DAPI (blue). DAPI-stained nuclei were less than 5% positive for GFAP in OPC cultures derived from independent mixed glia cultures. Data presented with mean and SEM (n=3 wells).

Primary cultures were then stained for markers of OPCs. Cultures were found to be over 95% positive for NG2 when quantified in cultures from independent preparations (Fig. 5.10). In addition, primary OPCs also expressed the early stage oligodendrocyte marker A2B5. It was quantified in cultures derived from a single isolation of OPCs indicating that more than 80% of these cells were positive for A2B5 (Fig. 5.11).
Figure 5.10. Cultures of primary OPCs were consistently NG2-positive. DAPI-stained nuclei were more than 95% positive for NG2 in OPC cultures derived from independent mixed glia cultures. Data presented with mean and SEM (n=3 wells).
Primary OPCs were seeded into 96 well plates, cultured in differentiation medium over 72 hours and assessed by brightfield imaging. As similarly observed with O2A-myc cells, a clear progression in morphology could be observed (Fig. 5.12). These changes can be quantified to indicate the maturation status of the cell (Barateiro and Fernandes, 2014). As outlined in methods (chapter 2), primary oligodendrocytes were assigned into five categories: I) bipolar/tripolar cells with simple processes, II) cells with increased secondary processes III) cells with increased primary and secondary processes, IV) highly complex cells with extensive tertiary processes, V) terminally differentiated cells with evidence of membrane expansions outside the cell body. All categories were detected in differentiating primary OPC cultures within 72 h (Fig 5.12).
Figure 5.12. Morphology of primary oligodendrocytes during differentiation in 96 well plates over 72 h. OPCs transform from a simple bipolar cell type to highly-complex, ramified cells with extended membrane formations outside the cell body. These morphologies can be categorised on a scale of I-V and quantified to assess changes in oligodendrocyte maturation.

Likewise, immunofluorescence imaging showed a decrease in NG2 expression and the presence of MOG-positive oligodendrocytes after 3 days in differentiation medium (Fig 5.13). A feasibility study was conducted using the Janus automated cell culture system. The aim was to determine if the screening of compounds on primary OPCs could be automated using a screening platform designed for the automation of cell culture, drug treatment, fixation and staining processes and which had been proven to reduce variability compared to manual methods. Unfortunately, the semi-adherent nature of rat OPCs precluded the possibility of employing this system.
Therefore, screening of effects of UPR modulation on OPC differentiation was carried out in PLL- and laminin-coated 96 well optical-bottom plates in two independent experiments using six replicate wells for brightfield imaging and three replicates for NG2/MOG immunofluorescent quantification.

**Figure 5.13.** Primary OPCs express MOG after three days in differentiation medium (inset). OPCs were stained for NG2 (red), MOG (green) and DAPI (blue) after 3 days in differentiation medium. Scale bar = 20μm.

5.4.4 **Treatment with IRE1-inhibitor 4μ8C inhibits OL maturation in the absence of induced stress**

At all timepoints there was a significant effect of 4μ8C treatment on the morphological status of the cells (Fig. 5.14, 5.15). Treatment with 4μ8C was found to have no effect on cell density (Appendix, Fig. A5.1). After 24 h there were significantly fewer cells exhibiting the category II phenotype when treated with 2μM or 10μM 4μ8C, when compared to those treated with vehicle and a corresponding increase in category I phenotype was observed in cells treated with 10μM 4μ8C versus vehicle (Fig. 5.15, top). At 48 h, 2μM- and 10μM 4μ8C-treated cells had significantly fewer cells in category IV compared to vehicle and this effect persisted at 72 h, with a concomitant increase in the less mature category III (Fig. 5.15, middle
and bottom). This data indicated a significant inhibitory effect of 4μ8C on OPC differentiation, which was evident at all timepoints.

**Figure 5.14.** Effects of 4μ8C on oligodendrocyte morphology. Cells circled in white indicate immature cells. Cells circled in blue indicate more differentiated cells.
Figure 5.15. 4μ8C inhibits maturation of OPCs in the absence of induced stress. Inhibition of IRE1 signalling via 4μ8C inhibited OL maturation at all timepoints. * denotes significant differences between 2μM or 10μM 4μ8C v DMSO vehicle (single, double, treble = p<0.05, p<0.01, p<0.001). Statistical analysis was performed using Two-Way ANOVA for effect of treatment on morphology with Bonferroni post-test from a minimum of three fields per well from four separate wells per group representative of two independent experiments.
After 72 h a small but consistent proportion of oligodendrocytes in control medium exhibited high MOG expression throughout the cell body and processes. The majority of cells expressed MOG in the cell body and in pockets of membrane expansions (Fig. 5.16, inset). Although the number of MOG-positive cells was non-significant between groups, cells treated with 4μ8C never exhibited high MOG expression throughout the cell (inset). Similarly, although the number of NG2 positive cells was not different between groups, cell processes were increasingly NG2-positive in treated groups versus control wells (Fig 5.16).

Figure 5.16. NG2 and MOG expression in 4μ8C-treated cells versus vehicle. OPCs were treated with 2μM, 5μM or 10μM 4μ8C or vehicle (DMSO) for 72 h in differentiation medium. NG2 (red) expression was more pronounced in OPC processes with increasing concentration of 4μ8C. High MOG (green) expression was not observed in 4μ8C-treated wells and was restricted to the cell body and membrane expansions (insets). Scale bar = 20μm.
5.4.5 Differential effects of guanabenz on OL maturation are time and dose-dependent

Guanabenz had a significant effect on morphology status of the cells at all timepoints (Fig. 5.17, 5.18). After 24 h wells treated with 5μM guanabenz contained significantly more category II cells and significantly fewer category I cells, when compared to vehicle (Fig 5.18, top). This inhibitory effect was absent at later timepoints. At 48 and 72 h, 2.5μM guanabenz appeared to exert an inhibitory effect on maturation (Fig. 5.18, middle, bottom). There were no significant differences in cell density in vehicle, 2.5μM or 5μM guanabenz treated cells after 24 h (36.8±10.1, 31.0±7.6, 38.2±9.0 cells/FOV respectively). When cell density was assessed at 72 h, however significantly fewer cells were observed in the 2.5μM treatment group versus vehicle (31.8±11.1 vs 53.3±9.4), despite no difference in density being observed between 5μM guanabenz versus vehicle (50.6±9.1 vs 53.3±9.4) (Fig. 5.20).
**Figure 5.17.** Effects of guanabenz on oligodendrocyte morphology. Cells circled in white indicate immature cells. Cells circled in blue indicate more differentiated cells.
Figure 5.18. Effects of guanabenz on OL maturation are time and dose-dependent. Increased maturation was observed in the 5μM guanabenz group at 24 h but this effect was lost at later timepoints. Numbers of mature cells were significantly decreased by 2.5μM guanabenz at 48 and 72 h. * and # denote significant differences between 2.5μM and 5μM guanabenz vs H2O vehicle respectively (single, double, treble = p<0.05, p<0.01, p<0.001). Statistical analysis was performed using Two-Way ANOVA for effect of treatment on morphology with Bonferroni post-test from a minimum of three fields per well from four separate wells per group, representative of two independent experiments.
A consistent minority of cells expressed high levels of MOG in control wells (H₂O vehicle), but these were not observed in wells treated with guanabenz. NG2 expression was more pronounced in cells treated with H₂O vehicle compared with DMSO (Fig. 5.16, 5.19). The NG2 expression pattern was not observed to change between guanabenz and vehicle-treated wells. A reduction in cell density was observed in 2.5μM guanabenz wells after 72 h and appeared to predominantly affect NG2-low, MOG-expressing cells (Fig. 5.19, 5.20).

**Figure 5.19.** Expression of NG2 and MOG in guanabenz-treated cells versus vehicle. OPCs were treated with 2.5μM or 5μM guanabenz or vehicle (H₂O) for 72 h in differentiation medium. Scale bar = 20μm.

**Figure 5.20.** Cell density is significantly decreased in 2.5μM guanabenz-treated cells at 72 h. Cell density was the same between groups at 24 h. No differences were observed between 5μM guanabenz and vehicle-treated wells. One-Way ANOVA with Tukey post test from a minimum of three fields per well from four separate wells per condition. Data presented with mean and SEM, representative of two independent experiments; ns, not significant.
5.4.6 Combined treatment of guanabenz and 4μ8C impairs differentiation at 72 h

To investigate whether amplification of PERK signalling by guanabenz could counteract the effects of IRE1 inhibition, both compounds were also applied in combination. Preliminary results suggested combined treatment of 4μ8C and guanabenz had no effect on cell number compared with controls (Appendix, Fig.A5.2). No changes were observed in morphological status versus vehicle at 24 or 48 h (Fig. 5.21, top and middle). At 72 h fewer category IV cells were observed in treated cells versus vehicle and all others were unchanged (Fig 5.21, bottom). Similarly, no overt changes in NG2 or MOG expression was observed between treated and control wells (Fig. 5.22).
Figure 5.21. Combined treatment of 5μM 4μ8C and 5μM guanabenz exerted an inhibitory effect on OL maturation at 72 h. The previously positive effect of guanabenz on early differentiation was negated by the action of 4μ8C. * denotes significant difference between treated and vehicle at 72 h (p<0.05). Statistical analysis was performed using Two-Way ANOVA for effect of treatment on morphology with Bonferroni post-test from a minimum of three fields per well from four separate wells per group, from a single experiment.
**Figure 5.22.** NG2 and MOG expression in OPCs treated with 4μ8C and guanabenz versus vehicle. OPCs were treated with 5μM 4μ8C and 5μM guanabenz or equivalent volume of vehicle (DMSO/H2O) for 72 h in differentiation medium. Scale bar = 20μm.

### 5.4.7 Optimisation of ATF6 gene silencing using a cocktail of siRNAs

As specific compounds targeting ATF6 were not available, a gene silencing approach was employed. Optimisation was undertaken in O2A-myc cells treated with the ER stress-inducing agent tunicamycin to test the efficacy of ATF6 gene silencing. A cocktail of 3 different siRNAs against ATF6 or scrambled non-targeting controls were tested at three different concentrations using Lipofectamine RNAiMAX transfection reagent. The highest concentration (10nM) of both ATF6 and control siRNA was toxic to O2A-myc cells and these groups were excluded from analysis. When gene expression of ATF6 was evaluated no significant changes were observed between cells treated for ATF6 or control siRNA either at 0.5nM or 1nM concentrations (Fig. 5.23). Indeed, statistically significant increases in ATF6 expression were observed in the 0.5nM ATF6 siRNA and 1nM ATF6 siRNA groups versus cells treated with tunicamycin only. The lack of gene silencing efficacy meant it was not possible to pursue this line of study.
Figure 5.23. Effect of non-targeting control or ATF6 siRNA cocktail on mRNA expression in O2A-myc cells treated with tunicamycin. ATF6 siRNA cocktail failed to effect gene knockdown relative to non-targeting siRNA and was found to significantly increase ATF6 mRNA relative to cells with tunicamycin alone. Statistical analysis was performed on dCt values of triplicate flasks using One-Way ANOVA and Tukey’s multiple comparison tests. * and *** denote significant differences between treatments at 72 h ($p<0.05$ and $p<0.001$ respectively).

5.4.8 Effect of 4μ8C on UPR and myelin gene expression during OPC differentiation

As 4μ8C had the most pronounced effect on OPC maturation, primary OPC cultures were scaled up to test the effect of IRE1 inhibition on myelin and UPR gene expression during differentiation. OPCs were differentiated over 72 h in the presence of 2μM, 5μM or 10μM 4μ8C or vehicle in triplicate flasks. Treatment with 4μ8C induced a consistent dose-dependent decrease in mRNA levels for PLP, MBP and MOG (Fig. 5.24).
Differentiating OPCs treated with 4μ8C exhibited a dose-dependent decrease in myelin gene expression. PLP, MBP and MOG gene expression all decreased in response to 2μM, 5μM and 10μM 4μ8C versus vehicle after 72 h in differentiation medium. n=3 flasks per treatment group.

When mediators of the UPR, ATF6 and XBP1, were assessed no significant changes were observed between 4μ8C and vehicle-treated cells (Fig. 5.25, top). In particular there was no change in levels of XBP1s mRNA indicating that the effect of 4μ8C was not mediated via UPR manipulation. In accordance with this, there were no changes observed in mRNA levels of classical downstream UPR targets, GRP78, GRP94 or PDI (Fig. 5.25, bottom).
Figure 5.25. Treatment of differentiating OPCs with 4μ8C had no effect on UPR gene expression. ATF6, XBP1s and XBP1u mRNA levels (top) were unchanged in response to 2μM, 5μM and 10μM 4μ8C. Similarly, downstream targets of UPR signalling GRP78, GRP94 and PDI were also unchanged with respect to vehicle after 72 h in differentiation medium. n=3 flasks per treatment group.
5.4.9 Long-term 4µ8C treatment in organotypic cerebellar slice culture model of remyelination

The lack of efficacy of 4µ8C on its target was surprising given its reported specificity in the literature. Most reports employ this compound in the context of induced or high stress conditions however and it may be more likely to act non-specifically in lower stress conditions (Maly and Papa, 2014). As the UPR had been observed during myelination of cerebellar white matter tracts (chapter 4), organotypic cerebellar slice cultures were employed to test whether 4µ8C would act specifically in the context of physiological ER stress and whether this would have an impact on myelination of axonal tracts by oligodendrocytes.

Organotypic slice cultures were prepared from 10 day old pups and demyelinated after 7 days in culture with LPC. After peak demyelination (48h), slices were treated with 5µM or 10µM 4µ8C or vehicle every second day until 14 days after LPC-induced demyelination. Despite long-term treatment, gene expression levels of XBP1s was not different between 4µ8C-treated and control slices in three independent experiments (Fig. 5.26). Similarly, XBP1u mRNA was also unchanged. In this paradigm there was no effect of 4µ8C on MOG at 14 days, in contrast to the effects observed on purified oligodendrocytes (Fig. 5.26, bottom).
**Figure 5.26.** Long-term treatment of organotypic cerebellar slices with 4μ8C after demyelination did not alter levels of XBP1 or MOG transcripts. No changes in mRNA expression of XBP1S (spliced) or XBP1U (unspliced) nor of the late stage myelin marker, MOG, were observed at 14 days post LPC. Three replicate wells were harvested per treatment group each containing three slices per well. Values are the means of three independent experiments.
5.5 DISCUSSION

Having observed UPR activation during active myelination \textit{in vivo}, this study aimed to more accurately identify which aspects of oligodendrocyte differentiation and myelination might be regulated by the UPR, and which arms of this signalling pathway may be important. A range of models were employed to address the impact of pharmacological inhibitors of the UPR on oligodendrocyte differentiation.

O2A-myc cells are a cell line derived from rat oligodendrocytes immortalised by low level expression of \textit{myc} oncogene (Barnett and Crouch, 1995a). These cells have been the basis of a number of studies (Barnett and Crouch, 1995a; Barnett \textit{et al.}, 1995b, 1998; FitzGerald and Barnett, 2000; FitzGerald \textit{et al.}, 2003; Liang, Draghi and Resh, 2004; Williams \textit{et al.}, 2005; Kraskiewicz and FitzGerald, 2011). O2A-myc cells were validated by expression of the essential cell lineage transcription factor olig2, expression of specific markers of OPCs and myelin and temporal changes in their morphology in response to differentiation medium. The ability to fulfil this repertoire of oligodendrocyte properties whilst being able to scale to volumes that would facilitate timepoint studies and gene expression analyses was valuable as the yield and expansion of purified primary OPCs can be poor.

The capacity of O2A-myc cells to mature under differentiating conditions was compromised however. They were not observed to form expanded myelin membranes, only weakly expressed MBP and lost viability after 5 days in differentiating medium. The original validation of O2A-myc cells confirmed their capacity to mature by A2B5-GalC+ expression and did not report myelin protein expression (Barnett and Crouch, 1995a). In a different model, expression of \textit{c-myc} under the MBP promoter resulted in widespread cell death and hypomyelination suggesting that \textit{c-myc} prevents terminal differentiation in oligodendrocytes (Jensen 1998). This study supports the findings of a previous report which found that overexpression of bcl-2 was necessary to overcome loss of viability during late-stage differentiation (FitzGerald and Barnett, 2000). Despite this, the early phase of O2A-myc differentiation proceeds normally and in this study, transcription of myelin-specific genes increased in a time-dependent manner and morphological changes associated with early maturation advanced as expected for up to 5 days. As gene
expression of UPR markers was unchanged during this timecourse, this suggests the UPR is not involved during the early phase of oligodendrocyte differentiation.

A more relevant model was required that would demonstrate late-stage differentiation and translation of myelin proteins. Primary OPC cultures were assessed for purity, morphological indicators and expression of early and late-stage markers in growth and differentiating conditions. Cells were over 90% pure and progressed through classical morphological stages of maturation. NG2 expression decreased as OPCs transitioned to more mature phenotypes in differentiation medium. One of the last known proteins to be expressed during myelination is MOG and indicates completion of the cells terminal differentiation program. Within 72 h in differentiation medium, a subset of oligodendrocytes exhibited high MOG expression throughout the cell, and the majority expressed MOG locally in the cytoplasm or in expanded areas of membrane outside the cell body. This time-point was chosen as it was unknown whether an inhibitory or pro-myelinating effect, if any, would be observed when screening agents acting on the UPR.

In this study, morphological indicators of OPC differentiation were analysed every 24 h and after 3d cells were stained for the OPC marker NG2 and the myelin protein MOG for assessing stage of oligodendrocyte maturity. These markers were unfortunately ineffective for assessing differences in oligodendrocyte differentiation in this assay. NG2 is a cell surface protein expressed on the cell body and processes of undifferentiated OPCs in development and adulthood, however its expression is rapidly lost as it becomes a post-mitotic, pre-myelinating precursor. MOG is a classically myelin-specific protein and considered a marker of terminal differentiation, but as one of the final products to be expressed during developmental myelination, typically 1-2d after PLP and MBP expression emerges, a longer assay time in culture may have been necessary in order to observe meaningful changes in numbers of MOG-positive cells. In retrospect, these markers represent the extremes of oligodendrocyte polarisation (Fig. 1.2), and the vast majority of cells in this assay lay in the spectrum in between, as indicated by low expression of both markers.

Although morphological indicators cannot specify lineage subsets defined by protein expression, they are a highly sensitive tool that gives additional information on cell complexity and facilitates repeated measures over time (Sperber and Arthur
McMorris, 2001; Liang, Draghi and Resh, 2004; Barateiro and Fernandes, 2014). In this approach, it cannot be excluded that any effects observed may be effects on the cytoskeleton or membrane organisation and not necessarily on cell maturation. Nonetheless such functional effects play a critical role in myelination and warrant analysis.

As different arms of the UPR appeared to be differentially activated during myelination in vivo (chapter 4), compounds specific for each signalling pathway were sought in order to dissect which, if any, were required for OPC differentiation. Specific compounds targeting ATF6 were not yet published at the time of the study. More general inhibitors targeting site-1 and 2 proteases were deemed unsuitable due to their essential function in cholesterol regulation. Indeed a recent report identified the essential function of SREBP processing in oligodendrocyte differentiation with the use of a selective site-1 inhibitor (Monnerie et al., 2016). As such, a gene silencing approach was employed. Optimisation was undertaken in O2A-myc cells treated with the ER stress-inducing agent tunicamycin to test the efficacy of ATF6 gene silencing. A cocktail of 3 different siRNAs against ATF6 or scrambled non-targeting control siRNAs with Lipofectamine RNAiMAX transfection reagent were tested at three different concentrations under RNase-free conditions in serum-free and antibiotic-free media whilst the cells were in growth phase. The use of an immortalised cell line in growth phase under mild ER stress was specifically chosen in order to boost chances of transfection and of observing gene knockdown. Although these conditions were expected to be more favourable than if undertaken in primary OPCs, still no differences in gene expression were observed between ATF6 siRNA and control siRNA-treated cells. A failure of transfection efficiency cannot be ruled out as this was not specifically assessed. A positive control siRNA targeting a non-essential HKG such as cyclophilin B, or a fluorescent indicator of transfection such as siGLO RNAi controls, would aid in determining whether efficient siRNA delivery to the nucleus occurred in the range of conditions used. This requires to be addressed first in order to establish optimal siRNA concentration or volume of transfection reagent, or whether transfection failure was due to the siRNA sequences used. Recent reports have identified the rough ER as the nucleation site for RISC signalling and siRNA-mediated cleavage of mRNAs. Furthermore, cell stress signalling, including that of ER stress, impairs this endogenous gene silencing
activity through sequestration of components of the RISC complex into stress granules (Stalder et al., 2013; Emde and Hornstein, 2014; Barman and Bhattacharyya, 2015). Indeed pre-treatment with Brefeldin A, an inhibitor of anterograde trafficking and inducer of ER stress, accelerated the rate of siRNA-mediated gene knockdown in HeLa cells and reduced the IC50 (concentration required to effect 50% knockdown at 24h) of the siRNA treatment ~10 fold (<0.2nM) when target gene expression was compared to DMSO vehicle control (Stalder et al., 2013). The siRNA in this study targeted Sjogren syndrome antigen B (SSB), which is ubiquitously expressed in all tissues, but was not compared with a control siRNA. Given the now recognised effect of ER stress on increased RIDD-mediated RNA decay, it would appear important to rule out that accelerated gene-knockdown was not a non-specific effect caused by Brefeldin A-induced ER stress. It is not yet known whether physiological ER stress or other chemical inducers, such as tunicamycin as used here, also have the same effect. Nonetheless, it demonstrates that much wider siRNA concentration ranges may need to be used in order to employ gene silencing approaches to the study of the UPR, and that much still remains to be learned about the interplay of the ER and gene silencing during stress and homeostasis. Fortunately, new chemical inhibitors or boosters of ATF6 activity have recently been developed, that are claimed to be specific, may overcome this issue in future (Gallagher et al., 2016a; Gallagher and Walter, 2016b).

Specific inhibitors of PERK and IRE1 signalling pathways and their mechanisms of actions have been well-established for a longer period. Guanabenz directly binds to GADD34 and blocks formation of the stress-induced eIF2\(\alpha\) phosphatase complex, GADD34–PP1C. In this way, eIF2\(\alpha\) phosphorylation is prolonged during stress but homeostatic regulation of eIF2\(\alpha\) is not affected (Hetz, Chevet and Harding, 2013). Guanabenz effects an extended blockade of protein translation and increased upregulation of downstream targets of PERK specifically in the context of ER stress. In addition however, guanabenz is an FDA-approved \(\alpha_2\)-adrenergic agonist used for the treatment of hypertension and off-target effects must be considered, particularly in the context of CNS disorders. This agent has been specifically used at identical concentrations on rat OPCs by the Popko group in relation to the ISR (Way et al., 2015). In this study, OPCs were seeded in differentiation medium and after 24 h cells were treated with IFN\(\gamma\), with or without, guanabenz for 48 h. Treatment with 5
μM guanabenz alone caused no change in viability of differentiating OPCs and similarly in this chapter, there was no difference in cell number between 5 μM guanabenz and control. Guanabenz was found to decrease IFNγ-induced apoptosis in differentiating OPCs. The ratio of peIF2α to eIF2α was assessed in these cells at 0, 4, 12, 20, 28 and 48 h. In the IFNγ-treated groups, levels were observed to increase and then decline over 48 h and a difference was observed only at the 20h timepoint, with stronger peIF2α levels detected in IFNγ- and guanabenz-treated cells compared to IFNγ alone. The effect of guanabenz alone was not studied in this experiment despite peIF2α being detectable in untreated cells after seeding in differentiation medium overnight (time 0) and at 48 h, suggesting an active integrated stress response was in effect. Although the authors continually express the hypothesis that myelinating oligodendrocytes are susceptible to ER stress, unfortunately a complete timecourse was not analysed in untreated cells to assess whether peIF2α levels changed during OPC differentiation in vitro.

Guanabenz was also applied to IFNγ-treated cerebellar slice cultures in the same study. Similarly to the model used here, parasagittal cerebellar organotypic slice cultures were used from P11 Sprague Dawley rats. This model is typically used for remyelination experiments (Birgbauer, Rao and Webb, 2004) as the cerebellum already contains myelinated axons at P10-11 (as observed in Fig. 4.2) which increases during a 7d recovery period at which time demyelination can be induced. In this study however, slices were treated 2d after preparation with IFNγ with or without guanabenz, a timepoint when myelination would be expected to be ongoing and partially complete in some areas. In this model, guanabenz is said to inhibit IFNγ-induced hypomyelination but this effect is unclear as IFNγ alone was observed to destroy the general architecture of the tissue when analysed by EM. The resulting hypomyelination may have been due to axon loss, OPC and/or mature oligodendrocyte loss, demyelination, differentiation blockade or a combination.

IFNγ has shown differential effects across the oligodendrocyte lineage. In vitro, IFNγ did not affect proliferation in early differentiating OPCs but increased PERK signalling and apoptosis. OPCs that were allowed to mature for several days before IFNγ exposure were more resistant, however, they showed decreased myelin gene expression prior to cell death by necrosis after 4-7d (Baerwald and Popko, 1998).
Effects on oligodendrocytes have also been investigated in transgenic mice in which IFNγ expression under the GFAP promoter could be controlled with doxycycline. It revealed that CNS expression of IFNγ during development reduces oligodendrocyte cell number and causes hypomyelination (Lin et al., 2005). These deficits were more severe in heterozygous PERK+/- mice and less severe in animals with enhanced PERK signalling in oligodendrocytes (Lin et al., 2005, 2008). Similarly, when guanabenz was administered daily from P7 onwards, a significant improvement in oligodendrocyte cell number and myelination was observed in these animals compared to vehicle (Way et al., 2015). Additionally, guanabenz treatment delayed the onset of EAE and at 8mg/kg concentration it was observed to reduce disease severity. Samples were further analysed at PID 15, a timepoint in which onset had initiated in WT animals but not in guanabenz-treated mice. The proportion of pIF2α+ mature oligodendrocytes was the same in EAE animals with and without guanabenz treatment but CHOP was only detected in spinal cord lysates of vehicle-treated EAE animals. This perhaps should not be surprising as EAE and MS lesions are known to upregulate CHOP and no lesions were present in guanabenz-treated animals at this timepoint (Mháille et al., 2008; Cunnea et al., 2010; Ní Fhlathartaigh et al., 2013). When immune cells were analysed it was shown that guanabenz had altered cytokine levels and reduced microglia and T cell frequency and distribution in the CNS. In another adoptive transfer model of EAE, guanabenz similarly reduced disease onset and severity. This model showed that guanabenz had not affected early infiltration of T cells but potently induced CD4+ cell death in the CNS at 10 d post transfer (Way et al., 2015). These favourable pleiotropic effects from a pre-approved FDA drug make guanabenz an exciting candidate for the treatment of MS and a clinical trial is currently underway (NCT02423083). Specific effects of guanabenz on OPC differentiation have still not been conducted up to now however.

Guanabenz appeared to show differential effects on OPCs at different concentrations. At 24 h, 2.5µM guanabenz had no effect on differentiating OPCs but at 48 h and 72 h, significantly higher percentages of immature cells were present in comparison to vehicle-treated cells which displayed later stages of differentiation. In addition, cell density did not increase between 24 h and 72 h in cells treated with 2.5µM guanabenz in contrast to vehicle and this appeared to be reflected in the notable reduction of MOG-positive stained cells. No differences in cell density were
observed in 5μM guanabenz-treated cells at either timepoint compared to vehicle. This supports the findings of Way et al., which reported no effect of 5μM guanabenz on viability of differentiating OPCs assessed by propidium iodide staining after 48 h (Way et al., 2015). Cells treated with 5μM guanabenz showed significant delay in differentiation only at 24 h and this effect disappeared at 48 and 72 h. Way et al., showed eIF2α was phosphorylated in OPCs plated in differentiation medium overnight and at 48h later. As suggested here, the prolonged phosphorylation of eIF2α by 5μM guanabenz in differentiating oligodendrocytes could potentially inhibit differentiation at early timepoints but this effect is transient. This may be due to compensatory mechanisms involving the UPR or ISR as both are homeostatic responses with multiple signalling mechanisms. In this culture model, the cell density of OPCs continues to increase until 48 h after induction of differentiation. The findings in this chapter indicate that 2.5 μM guanabenz may block cell growth and inhibit the proportion of OPCs that reach late stage differentiation at 72 h. Repeat studies are needed including toxicity testing to assess if selective cell death occurs in more mature cells but this may suggest a potential inhibitory effect of guanabenz on cell proliferation as well as delayed differentiation. As oligodendrocyte maturation was the focus of this study, effects of guanabenz on OPCs under growth conditions were not tested. This merits further attention as thus far direct effects of guanabenz at earlier and later stages of oligodendrocyte maturity are unknown and, in early differentiating OPCs, only the protective effect from exposure to IFNγ has been reported without regard to whether it affects differentiation.

As XBP1s is a critical mediator of IRE1 signalling and a transcriptional regulator of the UPR involved in membrane and lipid synthesis, an inhibitor of IRE1 RNase activity was chosen to block XBP1 activation. The umbelliferone 4μ8C was the most potent inhibitor to emerge from a high through-put functional screen of 238,287 compounds (Cross et al., 2012). This salicylaldehyde-based compound binds to a conserved lysine residue in the active site of the IRE1 RNase domain and prevents XBP1 splicing and RIDD activity by rendering it inflexible. 4μ8C had no measurable toxicity in wild-type MEFs up to 128μM and had IC50 values in the nanomolar range (Cross et al., 2012). The effect of 4μ8C on XBP1 splicing have been consistent across multiple cell types including T cells, pancreatic B cells and
various cancer cell lines (Cross et al., 2012; Cojocari et al., 2013; Kemp et al., 2013; Raina et al., 2014; Zhang et al., 2014; Bright et al., 2015; Gupta et al., 2015). The vast majority of such studies employ 4μ8C under conditions of ER stress induced by agents such as DTT, tunicamycin or thapsigargin or through mutant expression of misfolding proteins. It has been used to target macrophages in two inflammatory, in vivo models (Qiu et al., 2013; Tufanli et al., 2017). The effect of 4μ8C has been reported on T cells during TCR stimulation but studies analysing physiological stress are the exception (Kemp et al., 2013).

In this study, 4μ8C did not alter cell numbers but was observed to inhibit OPC differentiation and reduce myelin gene expression in a dose-dependent manner. Critically however, there was no significant change in the expression of spliced XBP1 indicating this may be a non-specific effect or that this effect may instead be mediated through RIDD activity if XBP1s levels are not dynamic in this system. The gene expression of folding proteins GRP78, GRP94 and PDI was also unchanged. Other reports have demonstrated that levels of chaperones are maintained in the presence of 4μ8C but levels of more selective XBP1 targets such as Derl1, Edem1, Erdj4 and Erdj6 are decreased and represent more appropriate targets to support XBP1s inhibition (Cross et al., 2012; Gallagher et al., 2016a). Given that purified OPCs did not engage in myelin wrapping in culture and that indicators of physiological ER stress had been observed in cerebellar tracts during myelination, the effects of 4μ8C were tested in organotypic cerebellar slice cultures. In this model, slices were demyelinated by LPC and allowed to recover for 12 days in the presence or absence of 4μ8C. In this system no changes were observed in levels of MOG or levels of XBP1u and XBP1s. Although 10μM 4μ8C is the most commonly used concentration for significant and sustained knockdown of XBP1s in many cell types, further optimisation may be needed to select the optimal concentration and timepoint for use in each culture model. Compensatory mechanisms cannot be ruled out but the lack of exogenous stress induction may be an important factor in assessing the efficiency of 4μ8C. The active RNase site of IRE1 is shallow and polar and the efficacy of salicylaldehyde-based compounds is believed to be driven by electrophile reactivity rather than binding affinity (Maly and Papa, 2014). The efficiency of this compound in the context of low physiological stress may differ from that observed during short-term induction of artificially high stress.
Overall, this study has provided preliminary data on the impact of UPR signalling on oligodendrocyte maturation and highlights important aspects to take into account in future studies. UPR signalling does not appear to be necessary for myelin gene expression and early maturation of oligodendrocytes in vitro. To determine how oligodendrocytes handle increased ER processing load during myelination requires models that allow the full myelination program to be executed. The models used in this chapter do not facilitate myelin membrane expansion and wrapping as occurs in vivo which are likely to be the phases when the ER of oligodendrocytes is most under stress. Using such in vitro models to screen the effect of UPR drugs may be inappropriate. In addition, gene silencing techniques are complicated by the intricate relationship between the ER and endogenous RISC signalling. Nonetheless, effects were observed with both guanabenz and 4μ8C, and have emphasised that the UPR signalling pathway merits further attention in myelination.
CHAPTER 6

DISCUSSION
6.1 DISCUSSION

The underlying processes of myelination are studied in the interest of basic science as well as in the hope that increased biological understanding of oligodendrocyte development could help advance the field of remyelinating therapeutics. Although the oligodendrocyte was first distinguished in 1899 (Robertson, 1899), rudimentary aspects of oligodendrocyte biology have remained obscure until recent years. How myelin wrapped around an axon could only be speculated upon until recent advances in sample preparation and electron microscopy could reveal the true ultrastructure of the myelin sheath (Snaidero et al., 2014). Rather than being a stationary accessory for axonal conductance, dynamic remodelling of the mature myelin sheath has confirmed the role of myelin in brain plasticity and learning (Chang, Redmond and Chan, 2016). The revelation that metabolic products are transferred between the axon and myelin internode has opened an entirely new integral function of mature oligodendrocytes (Morrison, Lee and Rothstein, 2013). The adult OPC population represents the largest life-long population of cycling precursor cells in the CNS and their responsiveness to injury not confined to demyelination points to further roles that remain to be uncovered (McTigue and Tripathi, 2008). In addition, improved culture models and genetic tools have made feasible functional assays and high-throughput screens for the validation of signalling pathways involved in oligodendrocyte differentiation. These rapid advances have emboldened efforts to establish the first remyelinating therapeutics, as lesion repair remains a continued unmet need of people affected by MS. Promisingly, the first clinical trials testing pro-myelinating therapies are already underway, including clemastine which has already reported subtle improvements in visual evoked potentials in MS patients affected by optic neuritis (NCT02040298).

This project aimed to analyse the potential role of the UPR in the differentiation of OPCs to mature oligodendrocytes. The sizeable synthetic burden placed on oligodendrocytes during myelin membrane manufacture has been widely appreciated, with the total membrane expansion representing a surface area up to 3,000-6,000 times larger than that of the cell body (Pfeiffer, Warrington and Bansal, 1993; Brady et al., 2005; Baron and Hoekstra, 2010). Unlike other tissues, all brain cholesterol, of which 80% is found in myelin, must be synthesised locally (Saher and Stumpf, 2014). This lipid-rich structure also contains integral membrane proteins
that are critical for myelin integrity and axonal health (Griffiths et al., 1998; Garbern et al., 2002; Lappe-Siefke et al., 2003). The ER is an essential cellular organelle in all eukaryotes for the synthesis of proteins, cholesterol and other lipids. The ultrastructural attributes of oligodendrocyte differentiation include a complete reorganisation of the endoplasmic reticulum. Beginning with short scattered cisternae throughout the cytoplasm of OPCs, elongated, parallel ER stacks are assembled at the base pole of thick processes during the active myelination phase. In mature oligodendrocytes, the cisternae shorten once again and tend to collect circumferentially around the nucleus (Peters and Folger Sethares, no date; Vaughn, 1969; Mori and Leblond, 1970; Vaughn and Peters, 1971; Parnavelas et al., 1983; Meinecke and Webster, 1984; Monzon-Mayor et al., 1990). These changes suggest that, as the oligodendrocyte lipidome and proteome is effectively reprogrammed during differentiation, these changing needs are met by a dynamic and responsive ER. This concurrent facet of oligodendrocyte differentiation may play an important role in facilitating myelin synthesis.

The UPR is the conserved signalling mechanism for homeostasis and adaptation to stress in the ER and for the induction of apoptosis when its capacity is overwhelmed. This mechanism was first recognised when upregulation of chaperones GRP78 and GRP94 was observed in response to the accumulation of malfolded proteins within the ER (Kozutsumi et al., 1988). In mammals, the UPR has developed into a three-armed co-regulatory signalling network mediated by three stress-sensing transducers - PERK, IRE1 and ATF6 - each affecting overlapping aspects of cellular function, e.g., the selectivity and rate of protein translation, protein processing capacity, ERAD, membrane biogenesis and induction of apoptosis (Ron and Walter, 2007). Since the discovery of these UPR initiators in the 1990s, their modes of activation, signalling mediators and targets have been extensively characterised individually. How these combine to integrate effective responses that reflect the intensity and duration of ER stress is still not well understood however (Hetz, Chevet and Oakes, 2015). Each of these UPR signalling pathways acts through distinct bZip transcription factors (ATF4, CHOP, XBP1s, ATF6(N)), which can homo- or hetero-dimerize with each other or separate transcription factors resulting in context- and cell-specific transcriptional outputs. One of the criticisms of the field has been the reliance on acute ER stress agents which induce simultaneous adaptive and apoptotic
responses that are not reflected physiologically (Hetz, Chevet and Oakes, 2015). In this work, an emphasis has been placed on analysing the activity of each of these arms concurrently at the level of UPR initiator activation and of downstream targets wherever possible.

Previous studies identified ER stress markers associated with an active UPR in the CNS lesions of multiple sclerosis and EAE (Mháille et al., 2008; Cunnea et al., 2011; McMahon et al., 2012; Ni Fhlathartaigh et al., 2013). The majority of reports on ER stress and the UPR are on tumour biology however, including brain tumours (Kraskiewicz and FitzGerald, 2012; Le Reste et al., 2016). Metabolic disorders, diabetes and neurodegenerative diseases have also been widely studied in the context of the UPR (Hetz, Chevet and Harding, 2013). The UPR has been a focal point of CNS proteinopathies such as Alzheimer’s disease and Parkinson’s disease (Hoozemans et al., 2009; Hoozemans and Scheper, 2012; Stutzbach et al., 2013; Valdés et al., 2014; van der Harg et al., 2014; Mercado et al., 2016; Wiersma et al., 2016). The involvement of the UPR in physiological stress has so far received attention in select “professional” secretory cells such as pancreatic β cells and B lymphocytes (Lee et al., 2005b; Zhang et al., 2006; Gass et al., 2009; Ma et al., 2010; Usui et al., 2012). In terms of CNS development, studies on the UPR to date are largely in vitro or have assessed only a single aspect or arm of the UPR. Nonetheless several studies support a role for the UPR in differentiation of specific neuron subtypes and in promoting indirect neurogenesis during cortical development (Hayashi et al., 2007; Zhang et al., 2007; Frank et al., 2010; Laguesse et al., 2015; Godin et al., 2016).

One of the first aims of this work was to profile UPR signalling during physiological development of the CNS in vivo and determine whether UPR activation and subsequent upregulation of chaperones were observed during myelination. Cerebellar white matter tracts were selected as the region of interest based on their long and widespread use in the literature. The profile of UPR expression in the normal rat cerebellum had not then been characterised although the UPR had been implicated in several examples of Purkinje cell neurodegeneration and had been observed within restricted parasagittal domains (Kyuhou, Kato and Gemba, 2006; Williams and Lipkin, 2006; Williams et al., 2007; Zhao et al., 2010). In order to rule out potential confounds presented by the intrinsic molecular profiles of distinct
parasagittal domains in the cerebellum, this characterisation was carried out in the
cerebellar cortical layers prior to the analysis of underlying white matter tracts. This
revealed new attributes of the mature and developing cerebellar cortex. Firstly, no
proteins associated with the UPR showed differential expression between the
parasagittal domains of the cerebellum. This indicates that increased expression of
these UPR markers during patterned Purkinje cell death, for instance during Borna
disease virus infection, is not due to inherently different basal UPR expression
profiles, and may instead be an indirect consequence of the pathology rather than a
contributory factor (Kyuhou, Kato and Gemba, 2006; Williams and Lipkin, 2006;
Williams et al., 2007). In addition, although the majority of markers were uniformly
expressed in the cerebellar cortex, Purkinje cells were observed to be either positive
or negative for XBP1 and pIRE1. This expression was apparently random and did
not subscribe to a symmetrically alternating pattern suggestive of parasagittal
domains. How this may relate functionally and whether it may relate to recently
active firing patterns is not known. In the analysis of UPR markers in the cortical
layers of the developing and adult cerebellum, a comprehensive picture of all
signalling arms was attained at each timepoint so that the acquisition of a cell-
specific, basal UPR profile could be observed, e.g. the strong expression of ATF6 in
Bergmann glia is first observed at P14 and into adulthood but this is not
accompanied by GRP78 or GRP94 chaperone expression. Conversely, these
chaperones are strongly expressed in the majority of neurons in the absence of
detectable ATF6 (Table 3.2). Although all the ER-resident proteins analysed in this
work will be present in all cell types, it is clear that their basal levels vary
dramatically from cell to cell. This indicates that the adoption of a sophisticated and
nuanced UPR profile under normal physiological conditions is a feature not just of
“professional” secretory cells, but of all cell types in the CNS. It was also shown that
certain UPR profiles were only necessary during developmental or intermediate
phases and this was most striking in the distinct pPERK-positive population in
postnatal cerebellum. Their abundance in the postnatal EGL and PWM gradually
reduced and disappeared by P21. pPERK and pIRE1-positive cells within the EGL
were most frequently observed in the premigratory, proliferating granule cell
precursors of the outer zone and within the PWM, these positive cells were also
frequently observed undergoing mitosis. The identity of the transient population in
the PWM needs to be confirmed but migrating GABAergic neuron progenitors are
likely candidates given the regional and temporal window they occupy. The proliferation of both GCPs and GABAergic neuron progenitors is driven by Shh and both are implicated as the cellular origins in subtypes of medulloblastoma, an aggressive childhood cancer (Fleming et al., 2013a; Wang and Wechsler-Reya, 2014). Investigating the functional role of this unique UPR profile may help reveal new aspects of the cell biology of these proliferative subgroups. As increased activation of IRE1 and PERK signalling pathways is associated with increased ER stress-induced apoptosis, it would be interesting to establish the sensitivity of these cell types to modulators of ER stress. Activated PERK signalling has been identified in human medulloblastoma and in murine models of Shh-type medulloblastoma (Lin et al., 2011; Ho et al., 2016; Stone et al., 2016). This group of studies analysed the effect on medulloblastoma formation when PERK signalling was reduced (PERK+/-), or increased (GADD34+/- and GADD34-/-) in transgenic mice. PERK+/- and GADD34+/- mice had decreased and increased incidence of medulloblastoma formation respectively but, surprisingly, GADD34-/- mice had the most reduced incidence of disease. All mutants had typical levels of cell proliferation, cell apoptosis, and angiogenesis in adult medulloblastoma tumours but at early stages of tumour formation, hyperplastic, pre-malignant lesions of GADD34-/- mice had increased numbers of apoptotic cells which is believed to result in reduced incidence of disease. This was not a result of CHOP expression however, a pro-apoptotic transcription factor associated with PERK signalling. Furthermore, although CHOP was greatly increased in adult GADD34-/- mice tumours, this did not result in an increase in apoptosis (Stone et al., 2016).

One limitation in these studies is that pre-malignant GCPs and tumour cells are not compared to normal granule cell development. Thus PERK signalling has yet to be recognised as part of normal GCP development in the EGL, and to our knowledge, neither has PERK signalling yet been reported in the postnatal PWM as part of normal cerebellar development as described in this thesis. This adds a second proliferative, PERK-signalling (and possibly Shh-responsive) cerebellar population that is directly affected in these models. A further limitation in these studies is that the PERK signalling pathway is analysed in isolation. The involvement of other UPR signalling pathways was not analysed and such compensatory mechanisms at play may have helped to unravel the seemingly conflicting effects observed. This thesis
has described activated IRE1 signalling in similar proliferative subpopulations in the EGL and PWM. Sustained or severe IRE1 activation promotes separate pro-apoptotic mechanisms via JNK/TRAF2 and RIDD. It would be highly interesting to know whether IRE1-activated cells are also observed in human medulloblastoma and murine models similar to PERK and whether combined targeting of these pathways could improve outcomes. Interestingly, when tunicamycin was delivered subcutaneously at P4, the cerebellum showed the greatest increase in cleaved caspase 3 levels in comparison to the cortex (slight increase) or liver (no change). IHC revealed this expression to be highest within the EGL, suggesting that GCPs are more sensitive to ER stress than other cells at this timepoint (Wang et al., 2015).

To analyse changes in UPR profiles during in vivo myelination, specific milestones in two anterior cerebellar tracts were established using early- and late-expressing myelin markers. The changes in glial cell populations, PWM cell density, positive-cell counts for each of six UPR markers were quantified for each timepoint and cell-specific expression of UPR markers was assessed by dual-immunofluorescence. There were several limitations in this study. Volumetric and stereological methods could provide more accurate estimations of cell counts. The analysis of cell populations present in the PWM would have failed to detect astrocyte progenitors that are GFAP-negative or GABAergic interneuron precursors (NeuN-negative) which are expected to be present in cerebellar PWM particularly in the second postnatal week. Glutamatergic UBC precursors in the PWM would also not have been detected but these are very rare in murine anterior cerebellar folia (Fig. 3.23). The olig2 lineage marker is not adequate to distinguish between OPCs and mature oligodendrocytes which would have made the data much more informative. Nonetheless patterns of UPR signalling could be observed to change as the PWM matured. In immature tracts pre-myelination (20.7% olig2+), few cells expressed activated UPR initiators or detectable chaperones. During active myelination (64.2% olig2+) cells expressed activated IRE1 and nuclear ATF6 and increased numbers of cells expressed typical UPR target chaperone proteins. Cells of adult white matter tracts (70.8% olig2+) no longer expressed activated UPR initiators but maintained high expression of non-nuclear ATF6 and ER chaperones. Furthermore, at P14 nuclear ATF6 and chaperone protein expression was directly observed in oligodendrocytes. This portfolio of UPR expression during normal in vivo
myelination had not been undertaken before. The majority of knockout mice for UPR proteins are lethal at embryonic or perinatal stages and so cannot provide evidence for or against a role in myelination (Table 1.2). It had previously been shown that oligodendrocyte PERK signalling was not required for myelination during development but that cell-specific activation of PERK in oligodendrocytes caused a phenotype with strongly similar features to Vanishing White Matter disease (VWMD) (Hussien, Cavener and Popko, 2014; Lin et al., 2014). This work supports the non-functional role of PERK for myelination in vivo, but adds that ATF6 and IRE1 signalling are specifically active during the myelination phase and are inactive post-myelination. This may indeed contribute to increased susceptibility to ER stress during this intensely active period. Interestingly, the administration of tunicamycin at P12 was shown to increase cleaved caspase 3 in the cerebellar PWM and DCN (i.e., during active myelination of these regions) whereas, when administered at P4 or P25, these regions were unaffected (pre- and post-myelination respectively) (Wang et al., 2015). Similarly, induced PERK activation in PLP-expressing cells caused severe oligodendrocyte pathology and hypomyelination during development but when applied in adult mice it significantly attenuated disease and demyelination by reducing oligodendrocyte apoptosis (Lin et al., 2013, 2014). These works suggest increased sensitivity of these cells to ER stress during active myelination. This study also highlights that mature oligodendrocytes express significantly elevated levels of ATF6, GRP78, GRP94 and PDI in vivo. Of all UPR-associated transcription factors, ATF6 is the most potent inducer of ER resident chaperones (Yamamoto et al., 2007; Adachi et al., 2008; Shoulders et al., 2013). Specific deletion of GRP78 in CNP-expressing cells resulted in reduced survival of mature OLs, correspondingly higher numbers of OPCs and death by P13 (Hussien et al., 2015). Furthermore, conditional deletion of GRP78 in PLP-expressing cells in adulthood resulted in apoptotic cell death and extensive demyelination, showing that it was essential for mature oligodendrocyte myelin maintenance and survival in vivo (Hussien et al., 2015). This is evidence of an essential functional role of GRP78 in oligodendrocytes which cannot be compensated by other ER-resident chaperones. Whether GRP94 or PDI are similarly essential for mature oligodendrocyte survival and maintenance and whether increased basal ATF6 levels are responsible or necessary for chaperone turnover warrants further study.
This study attempted to establish whether the UPR had an essential role in facilitating myelination. In the absence of being able to specifically modulate the UPR of oligodendrocytes in vivo or in ex vivo cultures, oligodendrocyte monocultures were treated with pharmacological modulators of the UPR in vitro. These models are commonly used to screen compounds for effects on myelination as canonical aspects of oligodendrocyte maturation can still be observed and these have positively identified drugs that were found to increase myelination in vivo (Najm et al., 2015; Lariosa-Willingham et al., 2016). Nonetheless these models have a number of drawbacks. Firstly, true myelination cannot be observed in monocultures as they cannot extend, wrap and form compact myelin sheaths around axons. For this reason, that chapter describes effects on oligodendrocyte maturation and differentiation instead of myelination. ER stress is typically present when the capacity of the ER is overreached, as hypothesised in the case of oligodendrocytes when dramatically increasing membrane proteins and lipids for myelination. Although differentiating OPC cultures expand membranes on the tissue culture surface, this is not close to the increase that occurs in vivo. Thus the levels of ER stress and UPR signalling within these cells may be lower than that in true physiological conditions. O2A-myc cells were observed to morphologically mature, lose NG2 expression and increase gene expression of PLP, MBP and MOG at 1, 3 and 5d in differentiation medium whereas UPR-associated genes (XBP1, XBP1s, ATF6, GRP78, GRP94, PDI) remained unchanged. This is in contrast to the UPR profile observed during in vivo myelination in chapter 4. Although these cells did not differentiate to the point of distinctly expressing myelin proteins, it allows the myelin gene expression program and early maturation changes to be uncoupled from the UPR. In support of this, in a report where site-1 protease activity was blocked in differentiating OPC cultures (which prevents cleavage of SREBPs for cholesterol synthesis but also blocks cleavage of ATF6 to its active nuclear-targeting form), impaired process extension was observed but there were no changes in oligodendrocyte transcription factors, GalC or PLP levels (Monnerie et al., 2016). In contrast, blockade of cholesterol synthesis in developing zebrafish showed that cholesterol was required for robust myelin gene expression and myelin wrapping (Mathews et al., 2014). This may point to differences between oligodendrocyte differentiation in vitro and in vivo, and potentially, a biphasic process in myelin membrane production. Indeed, transcriptional analysis of cells isolated from mouse
CNS has identified two consecutive myelin-forming oligodendrocyte (MFOL) subtypes from up to 12 lineage subtypes overall (Marques et al., 2016). Although both expressed genes for canonical myelin markers such as PLP and MOG, further gene ontology analysis of the top 50 differentially expressed genes in each subtype showed MFOL1 to be enriched for genes involved in lipid biosynthesis, membranes, methylation and axon ensheathment, whereas MFOL2 had genes associated with neurogenesis, membranes, cleavage of basic residues, calcium and synapses.

In this study, gene knockdown of ATF6 was attempted but failed. As outlined in chapter 5 discussion, the ER is directly involved in physiological and exogenous gene silencing and this approach was more complicated than first anticipated. Fortunately, although long believed to be “undruggable”, a new class of compounds claims to specifically inhibit ATF6α activation and its exit from the ER during stress, without altering ATF6β or SREBP activity (Gallagher et al., 2016a; Gallagher and Walter, 2016b). In addition, another high-throughput screening platform identified compounds to specifically promote induction of the ATF6 transcriptional program without globally activating the UPR (Plate et al., 2016). These new modulators have yet to be tested in different cell types and other physiological/pathological contexts but present welcome opportunities to probe this least understood arm of the UPR pathway.

When primary OPC cultures were treated with established UPR modulators, guanabenz and 4μ8C, impairments were observed in their ability to mature. Guanabenz at 5μM concentration exerted a transient impairment that was resolved after 72 h. However, 2.5μM guanabenz was found to reduce the proportion of differentiating cells as well as the expansion of OPCs in culture. Guanabenz has been proven to be protective of mature oligodendrocytes in various inflammatory models but its effects on myelination have not been examined before. Whilst preventing the need for remyelination is undoubtedly more preferable to having to repair a lesioned area in the first place, this important aspect of oligodendrocyte biology should still be investigated as guanabenz goes to clinical trial (NCT02423083). Considering that MS patients commonly have various lesion subtypes simultaneously (e.g., active, inactive, smouldering, remyelinating), different or composite needs may need to be met at different times.
The IRE1 RNase-domain inhibitor, 4μ8C, was found to impair oligodendrocyte maturation and decrease myelin gene expression in a dose-dependent manner. The fact that gene expression of UPR markers was unchanged indicates this was a non-specific effect and possibly that there may not have been sufficient IRE1 activity to inhibit in this assay. It supports the previous finding, that there is no correlation between myelin gene expression and UPR signalling during early maturation of oligodendrocytes. An *ex vivo* model of remyelination also failed to find downregulation of XBP1s but myelin gene expression was also not altered after 12 days of 4μ8C treatment. Although this inconclusive result is disappointing, it illuminates the needs that must be met to test such hypotheses regarding the role of the UPR and oligodendrocyte myelination. One critical need is for appropriate models that physiologically reflect the biological aspect we wish to study. Purified OPC cultures were used as opposed to mixed glia or neuronal co-cultures in order to establish specific effects of UPR modulation on oligodendrocytes. However their incapacity to expand and wrap myelin sheaths is likely to have failed to replicate the demands placed on the ER during myelination. A primary culture system using artificial nanofibres has been shown to facilitate extension, wrapping and compaction of myelin membranes (Bechler, Byrne and ffrench-Constant, 2015). This would facilitate the full myelination program of the oligodendrocyte to be studied in the context of the UPR. It would be ideal to study OPCs of relevant UPR gene knockout mice where possible. Most of these unfortunately are not viable (Table 1.2), but the development of cell-specific transgenic lines, inducible expression systems and the use of emerging classes of UPR modulators could help support this work. One such compound, subtilase, specifically cleaves GRP78 and thus induces ER stress without causing the unfolded protein build-up induced by other ER-stress causing agents (Wolfson *et al.*, 2008). This may be used concurrently during differentiation to observe the effects of a heightened UPR, or as a pre-conditioning agent, to observe whether this has differential effects at different stages of oligodendrocyte maturation. In addition to reaching the limits of ER processing capacity, many other types of insult (e.g. disruption to intracellular calcium, redox regulation, etc.) can also result in unfolded ER protein accumulation and this information may be useful in the context of oligodendrocytes in pathology. ER stress is known to be present in MS demyelinated lesions, so determining the sensitivity of
oligodendrocytes to this type of stress at different stages of the lineage could be important for evaluating cytoprotective and reparative strategies (Mhâille et al., 2008; Cunnea et al., 2011; McMahon et al., 2012).

In studying the UPR, some aspects were made difficult by the lack of available transgenic lines, the complexity of adopting a gene-silencing approach, ineffective antibodies for immunofluorescence (for IRE1 and XBP1) and limited drug targets of pharmacological modulators. Most of these aspects are already improving, however and could well be achievable in the near future as this field continues to expand.

This work suggests that in vitro maturation of oligodendrocytes may differ from that in vivo. Initial myelin gene expression and early differentiation processes appear not to be coupled to the UPR, but it is yet to be determined whether expansion and wrapping of concentric rolls of membrane lipids and proteins can occur without this response. If so, then determining an alternative mechanism for the expansion and reorganisation of the ER during myelin biogenesis merits further study. The traditional approach of studying the UPR using artificial in vitro systems and stress induction agents has identified many critical mediators and targets of this essential homeostatic response and continues to yield detailed information including dynamic pathway analysis (Walter et al., 2015). How applicable these mechanisms remain in other cell types and contexts, with individualised UPR profiles in more physiological settings, is unknown however. To take things forward requires a better understanding of cell-specific basal proteostasis mechanisms in order to better interpret pathological changes and develop more informed strategies. Targeting the UPR therapeutically will always be a challenge as it is intrinsic to every cell, but understanding cell-type differences in its signalling pattern and how it interacts with cell-specific transcription factors could prove very beneficial. For instance, ChIPseq data of oligodendrocyte transcription factors observed direct binding to the enhancer of ATF6 during oligodendrocyte maturation (Fig. 6.1, Prof. Q. R. Lu, Cincinnati Children’s Hospital Medical Center, personal communication).
Figure 6.1. Oligodendrocyte transcription factors bind to the enhancer of ATF6 at different stages of oligodendrocyte development (Prof. Q. R. Lu, personal communication). iOL, immature oligodendrocyte; mOL, mature oligodendrocyte.

How oligodendrocytes synthesise colossal amounts of complex membrane within a short space of time could be considered a degree of stress that would kill most other cells. It will be very exciting to observe how both the field of the UPR and the study of myelination, continue to advance in tandem to answer this remaining basic and mystifying question of oligodendrocyte biology.
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### OPC/O2A-myeloid cell culture reagents

**DMEM-BS**

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Cat No.</th>
<th>Component</th>
<th>Stock conc.</th>
<th>% (v/v)</th>
<th>200 ml</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma</td>
<td>D6046</td>
<td>DMEM</td>
<td></td>
<td>95</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>Sigma</td>
<td>P4458</td>
<td>Pen/Strep</td>
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<td>1X</td>
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<tr>
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<td>A4919</td>
<td>100X BSA stock</td>
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<tr>
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<tr>
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<td>100X N2</td>
<td>100X</td>
<td>1</td>
<td>2.0</td>
<td>1X</td>
</tr>
<tr>
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<td>L-Glutamine</td>
<td>200mM</td>
<td>1</td>
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</table>

Remove aliquots from -20°C freezer and thaw. Pool all 2ml aliquots and filter-sterilise before adding to a sterile media bottle or flask containing 190ml DMEM. The half-life of L-glutamine is approx..one month at 4°C and 7 days at 37°C therefore it should be added fresh. Label, date, initial and use +/- to indicate if supplements are absent. Circle the label when medium supplementation is complete. Store at 4°C for up to one month.

**OPC/O2A Growth medium (OPC/O2A-GM)**

OPC/O2A-GM consists of 25% B104-CM and 75% DMEM-BS. Thaw 1 aliquot of B104-CM (33.3ml) and sterile-filter into 100ml DMEM-BS, or 2 aliquots for 200ml DMEM-BS. Label, date, initial and use +/- to indicate if supplements are absent. Circle the label when medium supplementation is complete. Store at 4°C for up to one month. Alternatively, OPCs are maintained with DMEM-BS supplemented with 10ng/ml PDGF and 10ng/ml bFGF daily.

**OPC/O2A Differentiation medium (OPC/O2A-DM)**

OPC/O2A-DM is made up similarly to DMEM-BS but includes supplementation with T3 and T4.

<table>
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<th>Supplier</th>
<th>Cat No.</th>
<th>Component</th>
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<th>% (v/v)</th>
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<th>Final conc.</th>
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<td>1X</td>
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<td>Sigma</td>
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<td>100X T3 &amp; T4</td>
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<td>1X</td>
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<tr>
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<td>100X BSA stock</td>
<td>10mg/ml</td>
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<td>NAC</td>
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<td>63µg/ml</td>
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<tr>
<td>Biosciences</td>
<td>17502-048</td>
<td>100X N2</td>
<td>100X</td>
<td>1</td>
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<tr>
<td>Sigma</td>
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<td>L-Glutamine</td>
<td>200mM</td>
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</tbody>
</table>
Remove aliquots from -20°C freezer and thaw. Pool all 2ml aliquots and filter-sterilise before adding to a sterile media bottle or flask containing 188ml DMEM. The half-life of L-glutamine is approx. one month at 4°C and 7 days at 37°C therefore it should be added fresh. Label, date, initial and use +/- to indicate if supplements are absent. Circle the label when medium supplementation is complete. Store at 4°C for up to one month.

Concentrated stock solutions

Penicillin/Streptomycin (P/S)
Thaw a 100ml bottle of penicillin/streptomycin solution (Sigma P4458, -20°C) and aliquot into 2ml volumes for storage at -20°C.

10mg/ml Albumin from bovine serum (BSA)
Dissolve 500mg of BSA (Sigma A4919, 4°C) in a final volume of 50ml DMEM (D6046). Sterile-filter, aliquot and store at -20°C.

6.3mg/ml N-acetyle L-cysteine (NAC)
Dissolve 5g of NAC powder (Sigma A9165, 4°C) in 100ml of DMEM (D6046) and warm in a water bath (<15min). Solution will be yellow. Sterile-filter into a sterile bottle/flask containing a further 100ml of D6046 to yield a concentration of 25mg/ml. Transfer 12.6ml of this solution to a 50ml tube containing 37.4ml DMEM (D6046) to yield a concentration of 6.3mg/ml (100X) and repeat as necessary. Distribute into 2ml aliquots, label and store at -20°C.

N2 supplement (N2)
N2 supplement is used as required from the 5ml 100X product (Biosciences/Life Technologies, 17502-048, -20°C).

L-Glutamine (L/G)
Thaw 100ml bottle of 200mM L-glutamine solution (Sigma G7513, -20°C) and aliquot into 2ml volumes for storage at -20°C.
100X T3 and T4 stock
Make initial 1000X stocks of T3 and T4. Make a 6 mg/ml solution of T3 in 1 M NaOH solution. Add 250µl of this solution to 49.75ml of HBSS to make a 1000X T3 solution at 30ug/ml and repeat as required. Sterile-filter, aliquot and store at -20°C. Make a 1 mg/ml solution of T4 in 1 M NaOH and dissolve in a waterbath for 3-5 hours. For each 10ml, add 15 ml of DMEM to give a 1000X 400ug/ml solution. Sterile-filter, aliquot and store at -20°C. Add 5 ml of 1000X T3 and 5 ml of 1000X T4 to 40ml of DMEM to yield 50mls of 100X combined T3 and T4 stock and repeat as necessary. Distribute into 2 ml aliquots, label and store at -20°C.

Preparation of PDGF (Peprotech 100-13A)
Reconstitute the vial to 0.1mg/ml according to the specification sheet of the relevant Lot No. Centrifuge vial prior to opening. Mix up and down. Do not vortex. Make several master stock aliquots. Perform a 1 in 10 dilution of master stock to 10 µg/mL in sterile, chilled 0.2% BSA. Aliquot the PDGF working stock (e.g., 20 µL/tube) and snap-freeze in liquid nitrogen. Store aliquots at -80°C. Avoid repeated freeze-thaw cycles.

Preparation of bFGF (Peprotech 100-18B)
Check the spec sheet. Instructions for current Lot #051408-1 stipulate reconstitution in 5mM Tris, pH 7.6, to 0.1-1.0 mg/ml. Reconstitute the vial to 0.1mg/ml according to the specification sheet of the relevant Lot No., (e.g., for Lot #051408-1; 1mg is dissolved in 10ml of 5mM Tris, pH 7.6.). Centrifuge vial prior to opening. Mix up and down. Do not vortex. Make several master stock aliquots. Perform a 1 in 10 dilution of master stock to 10 µg/mL in sterile, chilled 0.2% BSA. Aliquot the bFGF working stock (e.g., 20 µL/tube) and snap-freeze in liquid nitrogen. Store aliquots at -80°C. Avoid repeated freeze-thaw cycles.

Preparation of 5mM Tris pH 7.6
Mix 50mM Tris-HCl and 50mM Tris base until the desired pH of 7.6 is reached. Dilute this solution 1 in 10 for a working concentration of 5mM Tris, filter-sterilise, label and store at -20°C if required.
Other reagent stocks:

**B104-CM**

See separate protocol for generation of B104 conditioned medium. B104-CM contains DMEM-BS harvested after 2-3 days in culture with B104 cells. It is stored in 33.3ml aliquots at -20°C or at -80°C for long-term storage.

**Trypsin**

Trypsin without EDTA is used to trypsinise OPC and O2A cells. 10X Trypsin (Sigma, T4674 -20°C, red solution) is thawed and aliquotted into 1-2ml volumes and stored at -20°C.

**SD mix**

Trypsin Inhibitor Type 1-S from Soybean, 100mg (SBTI, Sigma T6522)

DNAse (Sigma D4263)

BSA (Sigma A4919)

HBSS (Sigma H6648, without Ca²⁺/Mg²⁺)

Weigh out 600mg of BSA and 100mg Trypsin inhibitor and dissolve in 100ml of HBSS to make a 2X stock. Sterile-filter, aliquot, label and store at -20°C. Dissolve 2,000 units DNAse (1 vial from D6423) in 12.5ml HBSS to give a 2X concentration of 160U/ml. Aliquot, label and store at -20°C or if ready for use, add an equal aliquot of DNAse to an aliquot of 2X BSA/trypsin inhibitor in a 1:1 ratio. Filter-sterilise and store at -20°C or use immediately.

**Freezing medium**

Freezing medium consists of 90% HI-FBS and 10% DMSO.

**10% FBS, 1% P/S, DMEM**

When a vial of cells is recovered from -80°C or liquid nitrogen storage, cells are spun through 10% FBS, 1% P/S, DMEM.
Poly-lysine
Poly-D-lysine or poly-L-lysine may be used (such as Sigma P6407 or P4707). Aliquot poly-lysine at 1mg/ml (dilute in sterile water or other specified buffer if necessary). Alternatively, sterile poly-l-lysine solution (Sigma P4707) can be stored at 4°C up until the expiry date on the bottle. Add 100μl per 13mm coverslip, 1ml per T25, or 3ml per T75. Incubate at room temperature for minimum 5 minutes. Wash three times with sterile PBS or HBSS. Flasks can be used straight away, or left to air-dry in the TC hood before storing at RT for several weeks. Label the flasks with the date and ‘PLL’ to indicate that they are coated.

Mouse laminin stocks
Mouse laminin (Invitrogen 23017-015, size 1mg). Thaw laminin from -80°C slowly in the fridge at 4°C to avoid it forming a gel. 1mg of laminin is supplied in tris-HCl buffer at a concentration between 0.5-2.0 mg/ml. Once thawed, take note of total volume and hence concentration. Aliquot master stock into sterile tubes, store between 4°C and -20°C. Avoid repeated freeze-thaw and label with expiry in 6 months. When ready for use, dilute an aliquot to 10μg/ml working concentration in sterile distilled water.
**B - Table. A2.1.** Rat primer sequences and respective optimised concentrations and efficiencies.

<table>
<thead>
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<th>Category</th>
<th>Gene</th>
<th>Source</th>
<th>fwd/rev</th>
<th>Sequence (5’-3’)</th>
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<th>Primer conc.</th>
<th>Efficiency</th>
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<td>rev</td>
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<tr>
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<td>Reference</td>
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<td>rev</td>
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<td>MWG</td>
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<tr>
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<tr>
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<td>3μM</td>
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</table>
C - Figure A4.1. ATF6 and olig2 expression in P10 prospective white matter tracts in rat cerebellum. ATF6 expression detected by DAB single stain (top). Open and closed arrows show cytoplasmic and nuclear-localised expression respectively. and dual-chromogenic stain for ATF6 (DAB) and olig2 (liquid permanent red). Open and closed arrows show single and double-positive cells respectively. Scale bar = 100μm.
D - Figure A5.1. 4μ8C had no effect on cell density at 72h. Data presented with mean values and s.e.m.; unpaired Student’s t-test; n.s., not significant.

E - Figure A5.2. Combined treatment of 5μM guanabenz and 5μM 4μ8C had no effect on cell density at 72h. Data presented with mean values and SEM; unpaired Student’s t-test; n.s., not significant.

Differential activation of ER stress pathways in myelinating cerebellar tracts

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A B S T R A C T

Myelin production during brain development requires an increase in membrane protein and lipid production in oligodendrocytes and this primarily occurs in the endoplasmic reticulum (ER), an organelle which initiates the Unfolded Protein Response (UPR) when under stress. We hypothesise that the UPR is activated in white matter tracts during myelination in order to expand the ER capacity of oligodendrocytes.

Using early and late stage markers, critical myelination time points were identified by immunohistochemistry in developing rat cerebellum. These were correlated to peaks in ER stress signalling by staining for activated UPR transducers (pIRE1, ATF6 and pPERK) and associated downstream molecules (pERK, GRP78, GRP94, CHOP and calreticulin) in cerebellar tracts III and IV. Gene expression in developing cerebellum was assessed by qPCR.

Actively myelinating tracts were shown to have differential expression of pIRE1, PERK and ATF6 as well as UPR targets GRP94, GRP78 and PDI. Activated pIRE1-positive cells were widespread at P14 and P17 and at significantly higher numbers during myelination than at other stages. Nuclear-localised ATF6 (indicative of the active transcription factor) peaked at P10, concurrent with the initial phase of myelination. The percentage of cells positive for pPERK was less than 1% at postnatal ages but increased significantly in adult tissue. The downstream targets GRP78, GRP94 and PDI were significantly up-regulated at P17 compared to P7 and remained significantly elevated in adults. The majority of cells positive for these markers and ATF6 were oligodendrocytes as confirmed by dual-labelling. Although gene expression in the cerebellum for GRP78, GRP94 and PDI did not change significantly over time, ATF6 and XBP1s both showed significant fold changes between early and late timepoints.

This data helps promote understanding of events occurring during developmental myelination and may have implications for the development of reparative treatments in diseases such as multiple sclerosis.

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1. Introduction

Myelin components are synthesised, transported and inserted into the membrane in a regulated manner (Larocca and Rodriguez-Gabin, 2002). The principal site of synthesis for membrane proteins and lipids is the endoplasmic reticulum (ER). Ribosomes studded on the rough ER allow simultaneous translation and translocation into the ER lumen of proteins such as proteolipid protein (PLP), which alone comprises 50% of all myelin proteins (Eng et al., 1968). Meanwhile lipids are synthesised in the smooth ER including galactosylceramide, the precursor to myelin lipids galactolactocerebroside (GalC) and sulfatide (Blom et al., 2011). The simultaneous ensheathment of multiple axons by a single oligodendrocyte has been shown to occur in a short time-window (12–18 h) after which the cell loses capacity to myelinate (Watkins et al., 2008). It is estimated that an oligodendrocyte increases its surface.
area at a rate of 5–50 × 10⁴ µm²/cell/day during active myelination in the postnatal rat (Baron and Hoekstra, 2010). This could place a considerable burden on the endoplasmic reticulum (ER) of myelinating oligodendrocytes.

The ER ensures the conformational fidelity of its products through the action of ER-resident chaperones and folding enzymes and the targeted degradation of proteins that fail to attain their native state via the endoplasmic reticulum-associated protein degradation pathway (ERAD) (Meusser et al., 2005). The UPR is a homeostatic response that ensures that the capacity of the ER can meet demand or initiate apoptosis in the face of extreme stress. In mammals, three classical ER stress sensors are known to mediate the UPR: protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1), each containing a stress-sensing luminal domain and a cytosolic effector domain.

Upon activation, PERK dimerises and autophosphorylates and leads to phosphorylation of eukaryotic translation initiation factor 2α (p-eIF2α), which reduces the load on the ER by causing transient global translational arrest, apart from in select mRNAs with short open reading frames in the 5′ untranslated region. One such mRNA is that for activating transcription factor 4 (ATF4), which induces genes that aid amino acid metabolism, redox regulation and protein secretion (Harding et al., 2003). During severe ER stress, however, ATF4 induces the transcription factor C/EBP homologous protein (CHOP) which is associated with ER stress-induced apoptosis. IRE1 also autophosphorylates during ER stress and oligomerises in the ER membrane. This activates an endoribonuclease domain that splices X-box binding protein 1 (XBP1) mRNA to produce a potent pro-survival transcription factor for ERAD, membrane biogenesis, ER chaperones, redox enzymes and other cell-specific targets (Acosta-Alvear et al., 2007; Schroder and Kaufman, 2005; Wang et al., 2012). An additional role of IRE1 is its involvement in IRE1-dependent decay (RIDD), which degrades mRNAs destined for the ER and thus, alleviates protein load. These RIDD targets are predicted to have a similar secondary structure to the stem loop of XBP1 and contain a consensus sequence (CTGCAG) (Coelho and Domingos, 2014). During ER stress, ATF6 transits to the Golgi and undergoes proteolytic cleavage (Ye et al., 2000). The active transcription factor translocates to the nucleus where it strongly upregulates proteins which allow the ER to have a much greater capacity to process proteins (Wu et al., 2007; Yamamoto et al., 2007; Yoshida et al., 2001).

While the three UPR sensors are activated during pathological conditions (conditions that can be mimicked by addition of either thapsigargin or tunicamycin to cell culture systems), there is also known to be increased UPR signalling during normal physiological conditions where there is an increased load on the ER. Such a response leads to maintenance of homeostasis within the cell, rather than inducing cell-death, and often results in an upregulation of chaperones and folding enzymes. These include GRP78, GRP94, PDI and calreticulin. GRP78 (also known as BiP), is an ubiquitous chaperone protein that binds nascent proteins as they are being translocated into the ER and GRP94 (also known as HSP90B1, endoplasmia, gp96, and Erp95) is an ER chaperone with a much more restricted set of protein clients. PDI, a thiol-oxidoreductase folding enzyme, that generates disulphide linkages critical for the maturation of many ER-bound proteins, is required for lipid homeostasis as part of the IRE1-XBP1 pathway (Wang et al., 2012). Calreticulin, another ubiquitous chaperone, assists in the folding of newly synthesised glycoproteins and acts as a calcium buffer.

The laminar structure of the cerebellum allows identification of prospective white matter tracts before they are myelinated and has been a great aid to developmental studies of the morphology and immunohistochemical characteristics of oligodendrocytes during their maturation (Coffey and McDermott, 1997; Levine et al., 1993; Reynolds and Wilkin, 1988). However, the underlying mechanisms that facilitate this process are still being elucidated.

In this study, we have identified myelination milestones in cerebellar tracts III and IV according to the onset of production of myelin-specific proteins. We showed significantly increased activation of ATF6 and IRE1, but not PERK, prior to and during myelination. This activity returned to normal significance when myelination was complete. A significant increase in the expression of UPR targets GRP78, GRP94 and PDI was observed during active myelination and remained elevated in mature tracts in the absence of stress. We also show that these molecules are strongly associated with oligodendrocytes, but not astrocytes or microglia, in developing white matter tracts. When changes in UPR transcripts were monitored over the same time-period, significant increases in levels of spliced XBP1 and ATF6 were found. Further understanding of these processes may have implications in the development of treatments for demyelinating disorders such as multiple sclerosis.

2. Materials and methods

2.1. Animal husbandry and tissue processing

All experiments were approved by the National University of Ireland, Galway Animal Care Research Ethics Committee. Sprague–Dawley rat pups were bred from pregnant females obtained from Charles River (Harlan). Day of birth was designated postnatal day 0 (P0). Animals were sacrificed by decapitation at P7, P10 and P14, and by CO₂ asphyxiation at P17 and adult (>6 months). For chromogenic immunohistochemistry, pups were sacrificed before major maturation, tissue blocks were stored at −80 °C. Ten µm frozen sections were thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Dublin, Ireland) and stored at −80 °C until used.

2.2. Immunohistochemistry

All paraffin sections were dewaxed with xylene and rehydrated in graded dilutions of industrial methylated spirits (IMS) and water. Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide in 100% IMS for 20 min. Antigen retrieval was carried out in a pressure cooker in 0.01 M Tris–EDTA (pH 9.0) for 3 min for all antibody staining except MOG. Slides were incubated for 30 min at room temperature (RT) in blocking solution (5% normal goat serum in phosphate buffered saline (PBS)). Primary antibodies were diluted in blocking solution and incubated overnight at 4 °C and detected using biotinylated secondary antibodies and ABC horseradish peroxidase-labelled Vectastain Elite ABC reagent (Vector) or Dako Envision HRP as appropriate (Table 1). Diaminobenzidine (DAB) (Dako, Cambridgeshire, UK) was used as chromogen and all sections were counterstained in haematoxylin. Negative “no primary antibody” controls were included for each tissue block, for all stains (Supplementary Fig. 3). Details of antibodies and conjugates used for immunohistochemistry are shown in Table 1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdevneu.2015.08.002.
2.3. Imaging and quantification

Slides were scanned at 40× magnification with an Olympus VS120 digital scanner for quantification. For each of 3–6 animals, one field in the initial segment of lobule III and a second field in the initial segment of lobule IV (near base, Supplementary Fig. 1), was captured using OlyVIA software and converted to TIFF files. File IDs were reassigned and randomised using random.org to blind the assessor before analysis. White matter tracts (outlined by the internal granular layer) were defined and the area was measured in Fiji ImageJ. Total cell number and average cell density per time point were assessed by nuclear staining. Positively-stained cells for ER stress markers were manually counted and expressed as a percentage of total cell number to account for changes in cell density over time. Positive staining for GFAP and Iba1 was quantified using ImageJ and expressed as percentage area within delineated white matter tracts. Representative images were captured on an Olympus BX51 microscope.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jidevneu.2015.08.002.

2.4. Statistical analysis

Data were analysed using GraphPad Prism Version 5.0 software. Statistical analyses were carried out using Kruskal–Wallis tests with Dunn’s post-test or Two Way ANOVA with Bonferroni multiple-comparison analysis, as appropriate. Data are expressed as mean and the standard error of the mean (SEM). Differences were deemed statistically significant if \( p < 0.05 \).

2.5. Immunofluorescent dual-labelling

Slides were brought to room temperature and air-dried prior to incubation in ice-cold methanol for 20 min. All sections were blocked in 5% normal goat serum (NGS) in PBS for 30 min and incubated overnight with primary antibody diluted in 5% NGS at 4°C. The signal for rabbit polyclonal primary antibodies was amplified by incubating sections in a biotinylated swine anti-rabbit secondary antibody (1:400) for 30 min. Antibodies were visualised using streptavidin-Cy3 and goat anti-mouse Alexafluor 488 conjugates (see Table 1). All slides were mounted in Vectashield containing diamino-2-phenylindole (DAPI) (Vector) to allow visualisation of nuclei. Negative “no primary antibody” controls were included (Supplementary Fig. 3).

2.6. Quantitative PCR

Total RNA was extracted from snap-frozen cerebellum (\( n = 3 \)) using Qazol reagent according to manufacturer’s instructions and quantified using a Nanodrop ND-100 spectrophotometer. After DNase treatment, RNA was reverse transcribed using Superscript II Reverse Transcriptase and random primers (Invitrogen). Gene expression was analysed by quantitative PCR employing Fast SYBR Green Master Mix in a Step One Plus Real-Time PCR system (Applied Biosystems). PCR efficiency was determined for all primer sets. 18S was used as endogenous control and samples were normalized to \( P7 \) using the ddCT method. The sequences of PCR primers used are provided in Supplementary Table 1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jidevneu.2015.08.002.

3. Results

3.1. Milestones in the myelination of cerebellar tracts III and IV

Distinct stages of myelination were identified with respect to specific tracts in order to identify critical time-points for ER processing by oligodendrocytes. Progression of myelination in cerebellar lobes III and IV was qualitatively assessed using immunohistochemistry against early and late-stage myelin markers (Fig. 1). The DM20/PLP antibody is first detectable in immature oligodendrocytes making initial contact with axons. DM20 is a gene splice variant of PLP expressed on the surface of multi-branched, NG2-negative oligodendrocytes, representing the interim state from NG2-positive OPCs to fully differentiated oligodendrocytes (Kukley et al., 2010). PLP is a major structural protein specific to CNS myelin and is synthesised after ensheathment has begun (Hartman et al., 1982). Myelin oligodendrocyte glycoprotein (MOG) is one of the last myelin-specific proteins to be expressed. Therefore, the absence of both DM20/PLP and MOG in lobes III and IV was used to define tissue “pre-myelination” at \( P7 \) (Fig. 1). Faint MOG staining was observed at P10 alongside more abundant DM20/PLP, indicating the differen-
Fig. 1. Myelination of cerebellar tracts in postnatal rat cerebellum. Olig2 is a marker for the oligodendrocyte lineage (LH panels). PLP is present in compacted internodal myelin, whereas its splice variant, DM20, is expressed on the cell-surface and processes of immature myelinating cells as they begin to contact axons (middle panels). MOG, a late-stage myelin marker, is typically expressed 1–2 days after PLP (RH panels). Primary antibody staining was visualised by DAB immunohistochemistry and nuclei were counterstained with haematoxylin. Scale bar = 50 μm.
tiation of OPCs making axonal contact and the initial active phase of myelination. The area of staining of both proteins increased at P14 and P17 indicating accelerated myelination causing the expansion of WM tracts. In adult tissue, defined as age ≥6 months, staining with DM20/PLP and MOG antibodies was equally intense, and represented a timepoint in which mature oligodendrocytes would be involved in myelin maintenance.

3.2. Glial cell population changes during myelination

Cell density of white matter tracts in lobes III and IV changed significantly over time (Fig. 2a). There was a 30% increase in total cell number from P7 to P10 (7744 ± 743 and 10,110 ± 508 cells per mm²). From this maximum value, it reduced two-fold at P14 and ten-fold in adult (4405 ± 403 and 978 ± 153 cells per mm²) (Fig. 2a, LH panel). The density of oligodendrocyte lineage cells, as identified by olig2 expression, increased significantly from P7 to a maximum at P14 (1243 ± 110 and 2885 ± 76 cells per mm²). Thereafter it was reduced significantly in adult tissue (692 ± 31 cells per mm²) (Fig. 2a, middle panel). When expressed as a percentage of total cell number, olig2-positive cells were relatively constant at P7 and P10 (20.7 ± 1.8% and 21.5 ± 2.3%), then increased significantly to 87.2 ± 4.8% at P17 and stabilised at 70.8 ± 3.2% in adult tissue (Fig. 2a, RH panel). As reported previously, microglia undergo dramatic morphological changes during development (Ashwell, 1990). Our samples revealed amoeboid microglial cells with thickened processes at P7 and P10 which gave way to smaller cells with ramified branches, characteristic of “resting” microglia in older ani-

Table 2
Expression profile of ER stress-associated molecules in developing white matter.

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<th>P14</th>
<th>P17</th>
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<td>+</td>
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<tr>
<td>ATF6 (total)</td>
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</tbody>
</table>

Key: * = not detected; ** = 1–19%; *** = 20–39%; **** = 40–59%; ***** = 60–79%; ****** = 80–100% of cells.
mals (Supplementary Fig. 2). The percentage area detected by Iba1 staining almost tripled between P7 and P14 (11.0 ± 0.6–29.7 ± 3.4%) and decreased significantly in adult tissue (2.7 ± 0.6%) (Fig. 2b, LH panel). Similarly, astrocytes possessed enlarged cell somata and short filopodia in WM tracts pre-myelination and began to develop the characteristic branching of fibrous astrocytes only from P14 onwards (Supp. Fig. 2). Again, the percentage area of GFAP-positive cells more than doubled between P7 and P14 (25.1 ± 1.8% and 58.3 ± 8.3%) and was reduced significantly in adult tissue (13.7 ± 1.4%). There was a significant reduction in the area of GFAP-staining between P10 and adult (33.3 ± 1.9% vs. 13.7 ± 1.4%) and between P14 and adult (58.3 ± 8.3% vs. 13.7 ± 1.4%, Fig. 2b, RH panel).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jidevneu.2015.08.002.

3.3. ER stress sensors are differentially activated in white matter tracts undergoing myelination

To determine whether UPR signalling was present during myelination, the activation status of classical UPR initiators was analysed at each stage of WM tract development. Antibodies detecting phosphorylated PERK picked out isolated cells at P7, P10 and P14, (<1%) (Fig. 3a). This was followed by a significant increase in pPERK-positive cells in adult tissue although these were noted to be weakly-stained (9.2 ± 2.5% vs. 0.5 ± 0.3% at P14, Fig. 3b). Downstream indicators of PERK activation, peIF2α and CHOP, were not detectable at any age (data not shown).

In contrast, the percentage of phosphorylated IRE1-positive cells was low in white matter tracts before myelination, P7, but increased significantly to widespread levels at P14 and P17 (64.0 ± 13.6% vs. 45.0 ± 7.6%, Fig. 4a and b). Staining at these latter time-points was punctate in nature. In myelinated adult tissue, it returned to significantly lower levels (Fig. 4b). Detection of pIRE1 varied from a granular appearance to more diffuse cellular staining pattern.

During ER stress, ATF6 is proteolytically cleaved and the active transcription factor translocates to the nucleus. Here, nuclear-localised ATF6 (ATF6(N)) was observed at P7, P10 and P14, and was absent in older animals. A concurrent increase in non-nuclear ATF6-positive cells (ATF6(C)) was observed at these later time-points (insets, Fig. 5a). This shift between nuclear and non-nuclear staining in the population of ATF6-positive cells was significant between P10 and P17 (68.3 ± 11.4% vs. 0.0 ± 0.0% for ATF6(N)), and between P10 and adult (68.3 ± 11.4% vs. 0.0 ± 0.0% for ATF6(N)) (Fig. 5b, middle and RH panel). The change in the percentage of ATF6(N) stained cells was also significant with respect to the total cell population (P10, 19.1 ± 5.6% vs. P17 and adult both 0.0 ± 0.0%) (Fig. 5b, lower LH panel). Likewise, the increase in the percentage of ATF6(C)-positive cells in white matter tracts was significant at P10 and P14 vs. adult (7.7 ± 2.3% and 12.0 ± 5.7% vs. 46.2 ± 5.1%, Fig. 5b, lower RH panel).

3.4. UPR targets are significantly upregulated and remain elevated after myelination

The UPR upregulates the expression of ER-resident chaperones and folding enzymes in order to increase the processing capacity
of the ER. As the density of oligodendrocytes increased (Fig. 2), we observed increasingly abundant numbers of cells positive for UPR targets, the majority of which displayed the typical morphology of mature oligodendrocytes (Fig. 6a).

A continual increase in the percentage of GRP78-positive cells was observed over time and this was significantly different at P7 and P10 when compared to P17 and adult (5.7 ± 2.4% and 10.8 ± 2.1% vs. 43.4 ± 5.3% and 85.8 ± 14.4%) (Fig. 6b, LH panel). The number of cells positive for GRP94 was found to be significantly different between P7 and all other time points (P7 15.5 ± 1.3% vs. P10 32.7 ± 2.6%, P14 31.9 ± 3.6%, P17 55.8 ± 5.8% and adult 46.5 ± 8.4%) (Fig. 6b, middle panel). A significant increase in cells positive for PDI was observed between P7 and P10 vs. P17 and adult (1.9 ± 0.5% and 2.3 ± 0.3% vs. 21.2 ± 2.9% and 31.2 ± 4.7%) (Fig. 6b, RH panel). Detectable calreticulin expression, however, was not observed within white matters tracts in this data (not shown).

3.5. UPR signalling pathways are differentially activated prior to and during myelination

To reveal the relative contributions of each arm of the UPR at each time point, a 2-Way ANOVA and Bonferroni post hoc test was used to determine significant differences in the number of pPERK, pIRE1 and ATF6(N)-positive cells over time (Fig. 7a). A statistical correlation was found between time and the number of cells positive for activated UPR initiators (p < 0.0001 for each). Specifically, prior to myelination at P7, nuclear-localised ATF6 was observed in twice as many cells as activated IRE1 (697.0 ± 268 vs. 361.6 ± 218 cells per mm²), which was more abundant again than pPERK (104 ± 20.3 cells per mm²). During the early active phase of myelination at P10, both ATF6(N) and pIRE1-positive cells increased to ~2000 cells per mm² and were significantly higher than pPERK which was unchanged (p < 0.001). At P14 however, there was a 10-fold difference between the numbers of pIRE1 and ATF6(N)-positive cells (2875.6 ± 612.5 vs. 281.9 ± 153.2 cells per mm², p < 0.001). ATF6(N) was no longer detected after P14 while pIRE1-positive cells declined at a slower rate. At P17, significant differences persisted between pIRE1 and ATF6(N) (1183.7 ± 198.7 vs. 0.0 ± 0.0 cells per mm², p < 0.01) and between pIRE1 and pPERK (71.4 ± 0.0 vs. 53.3 cells per mm², p = 0.05). In post-myelination, adult tissue significant differences were no longer seen (Table 2).

When the same statistical tests were applied to detect variation in UPR targets at these time-points, a significant difference was also found and both time-point and UPR target each significantly influenced this (Fig. 7b, p < 0.0001 for all). Before the onset of myelination at P7, the number of positive cells for GRP94 was substantially greater than for GRP78 or PDI (1202.4 ± 97.4 vs. 443.1 ± 187.3 and 146.8 ± 37.2 cells per mm², p < 0.001 and p < 0.001 respectively). At P10, these differences were more dramatic and GRP94-positive cells were 3-fold higher than for GRP78 and 14-fold higher than for PDI (3030.4 ± 262.6 vs. 1087.6 ± 212 and 232.8 ± 32.5 cells per mm², both p < 0.001), despite GRP78-positive cells having doubled in number and also being significantly different to PDI (p < 0.01). PDI-positive cells did not increase substantially until P14 (952.0 ± 132.5 cells per mm²). No significant differences
Fig. 5. ATF6 expression in developing rat cerebellar tracts. (a) During myelination, ATF6-positive cells were primarily nuclear, indicative of the active transcription factor. Post-myelination, no ATF6-positive cells exhibited nuclear localisation. Positively-stained cells (brown) showed nuclear localisation at younger ages (P7, P10, P14) and was replaced by non-nuclear staining at later time-points (see insets). Primary antibody staining was revealed by DAB immunohistochemistry (brown) and nuclei were counterstained with haematoxylin (blue). Scale bar = 20 μm. (b) The total number of cells positive for ATF6 increased significantly between P17 and adult. From this population of ATF6-positive cells, the percentage with nuclear staining was significantly higher at P10 vs. P17 and adult. Similarly, the percentage of non-nuclear stained cells was significantly raised at P17 and adult vs. P10. The percentage of cells with nuclear-localised ATF6 was also significantly raised within the total cell population at P10 vs. P17 and adult. A significant rise in the percentage of non-nuclear ATF6 stained cells in the total cell population was observed between P10 and P14 vs. adult tissue. Data are mean ± SEM. (n = 3) *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 6. Expression of GRP78, GRP94 and PDI in developing rat cerebellar tracts. (a) The number of positive cells for these downstream targets increased after the activation of UPR transducers and remained elevated in adult tissue. Primary antibody staining was visualised by DAB immunohistochemistry and nuclei were counterstained with haematoxylin. Scale bar = 20 μm. (b) The ratio of GRP78 positive cells increased significantly between P7 and P10 vs. P17 and adult. Cells positive for the ER chaperone GRP94 increased significantly between P7 vs. P10 and P14 and increased further at P17 and adult. Cells positive for the folding enzyme PDI were significant at P17 and adult vs. P7 and P10. Data are mean ± SEM GRP78: n = 4 for P7, P14 and adult; n = 5 for P10 and P17. GRP94: n = 5 for P7; n = 4 for P10; n = 6 for P14; n = 3 for P17 and adult. PDI: n = 3 for all but P17 when n = 4. *p < 0.05, **p < 0.01, ***p < 0.001.
were detected between the UPR targets at P14, P17 or adult (Table 2).

3.6. ATF6(N) and its downstream targets are closely associated with oligodendrocytes

Cerebellar white matter tracts at P14 represent a time of flux in which oligodendrocytes, astrocytes and microglia can be observed in a mixture of polarised states i.e. OPC vs. mature oligodendrocyte and ‘activated’ vs. ‘resting’ phenotypes of astrocytes and microglia (Supplementary Fig. 2). To ascertain which lineages were expressing ATF6, we conducted dual-immunofluorescence staining with cell-specific markers. Olig2 expression is high in OPCs relative to mature oligodendrocytes where it is lower or sometimes absent (Kitada and Rowitch, 2006; Kuhlmann et al., 2008). ATF6 expression was localised both inside and outside the nucleus of olig2-positive cells (Fig. 8, LH panel). Non-nuclear ATF6 expression was also occasionally associated with microglia, but was absent in astrocytes (Fig. 8 middle and RH panels).

3.7. Downstream targets of UPR are highly expressed in oligodendrocytes

As GRP78, GRP94 and PDI are all strongly induced by ATF6(N) and to a lesser extent by spliced XBP1 (Shoulders et al., 2013) we assessed whether these ER folding proteins would be specifically up-regulated in oligodendrocytes in a period of active myelination. At P14, GRP78, GRP94 and PDI were almost exclusively associated with olig2-positive cells (Fig. 5, top row). Although the presence of positive cells that were olig2-negative was noted, GRP78, GRP94 and PDI did not appear to co-localise with GFAP or OX-42 staining (for astrocytes or microglia, respectively) (Fig. 9, middle and bottom rows). These cells may be associated with mature olig2low/absent oligodendrocytes (Kitada and Rowitch, 2006; Kuhlmann et al., 2008). GFAP-negative astrocytes or OX42-low positive microglia could also account for these cells. In addition, a minority of olig2-positive cells did not express GRP78, GRP94 or PDI at P14. These cells may represent immature oligodendrocyte precursors, given the high expression of these molecules in adult tracts populated by mature oligodendrocytes.

3.8. Gene expression profile of IRE1 and ATF6 signalling in developing cerebellum

Activated IRE1 and ATF6 regulate UPR transcriptional programmes through spliced XBP1 and cleaved ATF6 respectively. qPCR analysis revealed dramatically increased mRNA expression of spliced XBP1 between P7 and adult (approx. 150-fold). ATF6 gene expression also increased significantly between P10 and P17; however gene expression of UPR targets GRP78, GRP94 and PDI did not change significantly over time (Fig. 10).

4. Discussion

Although populated by oligodendrocyte precursors before birth, myelination is initiated only at P10 as cerebellar cortical neurons mature ((Reynolds and Wilkin, 1988) and Fig. 1). Cell density decreases once myelination is initiated due to volumetric expansion, substantial astrogial death and the apoptosis of OPCs that fail to myelinate (Altmann, 1982; Bandeira et al., 2009; Krueger et al., 1995). In our study, oligodendrocytes accounted for 20% of cells in prospective tracts at P10, rising to over 70% in adult tracts. In keeping with other reports, microglial morphology was seen to change from an early amoeboid morphology (P7–P10), to a ramified shape consistent with a resting phenotype (Ashwell, 1990; Salter and Beggs, 2014).

It is estimated that a postnatal rat oligodendrocyte increases its surface area at a rate of 5–50 × 10^3 μm²/cell/day during active myelination (Baron and Hoekstra, 2010). This could reasonably be expected to place an increased load on the ER, as it is responsible for the synthesis, processing and trafficking of membranes. Despite this, no study has yet compared the activation status of the three arms of the UPR, or the levels of downstream targets during developmental myelination in vivo.

Quantification of immunohistochemical staining revealed that detection of both nuclear ATF6 and pIRE1 are significantly increased prior to, and during, myelination. In contrast, PERK activation was minimal. This supports the findings of Popko’s lab, who have extensively investigated the role of PERK signalling as part of the integrated stress response (ISR) in oligodendrocytes. Although normal developmental myelination in oligodendrocyte-specific PERK-null mice has been described, these mice have exacerbated disease in EAE (Hussien et al., 2014). Indeed, stimulation of PERK signalling can significantly improve OL survival in EAE (Lin et al., 2013) and is now being investigated as a potentially therapeutic approach for inflammatory demyelinating diseases such as multiple sclerosis (Way et al., 2015).

Moreover, we were unable to detect pELF2α or the presence of transcription factor CHOP. Similarly, Lin et al. (2013) observed no expression of pELF2α in oligodendrocytes in normal adult spinal cord. Others also found CHOP staining to be undetectable in adult mouse white matter tracts, although low basal mRNA levels for
Fig. 8. ATF6 is activated in oligodendrocytes during myelination. ATF6 (red) is shown with cell-specific markers (green) and DAPI nuclear staining (blue) in white matter tracts of P14 rat cerebellum. Both nuclear and non-nuclear localised ATF6 expression is observed in olig2-positive cells. ATF6 is observed outside the nucleus in microglial cells indicated by OX-42. ATF6 was not associated with astrocytes revealed by GFAP staining. Arrows indicate ATF6 expression outside the nucleus (inactive) in oligodendrocytes and microglia. Arrowheads show nuclear staining indicative of cleaved ATF6 transcription factor in oligodendrocytes. Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this transcription factor were present in spinal cord homogenates (McLaughlin et al., 2007; Southwood et al., 2002). In our study, since the number of PERK-positive cells was so much lower than the number of developing oligodendrocytes that are known to be present, pPERK antibodies may selectively be staining an, as yet, unidentified small population of neural precursors. Because of the absence of detectable pelF2α or CHOP protein, we hypothesise that these cells could be undergoing pelF2α-independent modifications, possibly associated with autophagy. This follows from recent reports of PERK-dependent, but pelF2α-independent, regulation of autophagy via pAMK, HSP90 or pFOXO-beclin1 (Liu et al., 2015), and the commonly-reported need for functional autophagy in the normal development of cerebellar neurons (Marzban et al., 2014). Further investigation will be needed to confirm this.

Interestingly, activation of ATF6 and IRE1 was detected in white matter tracts at P7, before the appearance of myelin proteins. ATF6 expression peaked at P10, while pIRE1 peaked at P14. The percentage of cells positive for pIRE1 was significantly greater at P14...
and P17, when the rate of myelination is at its greatest, compared to time points pre- and post-myelination. Nuclear ATF6 was observed in cerebellar tracts at P7, P10 and P14, just prior to, and during, the initial phase of myelination, which precipitates a vast increase in membrane synthesis. ATF6 is known to increase phospholipid synthesis both independently, and in conjunction with, XBP1 mRNA (Bommiasamy et al., 2009). Furthermore, ATF6 activation has been reported in response to membrane protein expression in the absence of stress (Maiulo et al., 2011). Although studies using the ATF6<sup><s></s></sup>mouse show it to be developmentally-normal this may be due to compensatory functions by the ATF6<sup></s></sup>isofrom. This is further substantiated by the fact that the double knock-out (ATF6<sup></s></sup>−/−;ATF9<sup></s></sup>−/−) is embryonically-lethal (Yamamoto et al., 2007). Also, IRE1/XBP1 signalling in the brain has been shown to be beneficial in stress-resistance, aging and models of neurodegeneration (Mardones et al., 2015). The variety of proteins that have been reported to be targeted by ATF6 or IRE1 signalling, including those related to protein folding, trafficking and degradation (Shoulders et al., 2013), could be beneficial to the shifting protein and lipid requirements of oligodendrocytes as they myelinate.

The UPR in oligodendrocytes has been linked to various myelin disorders including multiple sclerosis, Charcot–Marie–Tooth disease, Vanishing White Matter disease and Pelizaeus–Merzbacher disease (PMD). PMD is an X chromosome-linked disease arising from mutations in PLP resulting in a range of phenotypes and severity. Accumulation of mutant PLP in oligodendrocytes results in a distended ER ultrastructure and chronic UPR activation (Barrie et al., 2010; Bauer et al., 2002; McLaughlin et al., 2007). Although mutant mouse models of PMD displayed species and strain variability, reduced disease severity and increased survival was found to be correlated with the expression of the UPR transcription factor CHOP in oligodendrocytes (Southwood et al., 2002). This indicated a cell-specific pro-survival function of CHOP and indicated a modified UPR in oligodendrocytes. The co-localisation of CHOP in oligodendrocyte nuclei of PMD patients further strengthened the clinical relevance of the UPR (Southwood et al., 2002).

With our data, this suggests that oligodendrocytes may utilise a specialised UPR to cope with exceptional synthetic demand, such as that arising during myelogenesis within developing white matter tracts. It may be that, in this situation, oligodendrocytes are employing a strategy similar to that used by differentiating B lymphocytes, where the three arms of the UPR are differentially activated, with a notable suppression of the PERK pathway (Ma et al., 2010). Since PERK signalling is known to result in a significant dampening of global protein synthesis, it is possible that a PERK-suppressing strategy could be beneficial in the case of active processes such as immunoglobulin production and myelination.

Changes in the levels of downstream targets of the UPR were also investigated. Although CRT was not detected, GRP78, GRP94 and PDI were all found to increase as myelination progressed and

**Fig. 9.** UPR target proteins strongly associate with oligodendrocyte cells. GRP78 (LH panels), GRP94 (middle panels) and PDI (RH panels) are labelled red and cell-specific markers green, with DAPI nuclear staining in blue, in white matter tracts of P14 rat cerebellum. GRP78 is expressed in olig2-positive cells but does not associate with microglia or astrocytes as revealed by OX-42 and GFAP staining, respectively. Similarly, GRP94 and PDI strongly associate with oligodendrocytes and are absent in microglia and astrocytes. Scale bar = 5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 10. UPR gene expression in developing rat cerebellum. (a) mRNA expression of spliced XBP1s increases dramatically between P7 and adult. ATF6 mRNA is significantly increased between P10 and P17. (b) mRNA expression of UPR targets GRP78, GRP94 and PDI show no significant change over time. Data are expressed in 2ddCT (n = 3). T < 0.05.

were significantly higher at P17 and adult vs. unmyelinated tissue at P7. The expression of all three proteins also remained elevated into adulthood. The chaperone CRT was not upregulated in myelinating tracts in our study. CRT acts as a major calcium buffer and as a partner in the calreticulin–calnexin cycle for the folding of glycoproteins in the ER and our group has previously shown upregulated CRT expression in EAE pathology (Ni Fhlathartaigh et al., 2013). Despite being a target of the UPR, CRT is not required for MOG expression (Jung and Michalak, 2011) and its function as a calcium buffer can be compensated for by GRP94 (Argon and Simen, 1999) suggesting it may not be required for myelination.

Although highest expression of GRP78, GRP94 and PDI occurred in mature tracts in the absence of UPR activation, high basal levels of such proteins are not uncommon. D’Souza and Brown (1998) showed constitutive expression of chaperones in developing and adult rat cerebellum and similarly we observed no significant change in mRNA levels over time. All three ER-resident proteins have been detected in myelin proteomic studies as well as cultured oligodendrocytes (de Monasterio-Schrader et al., 2012; Neri et al., 1997). Chromogenic staining for UPR targets and ATF6 was conspicuously associated with the cell morphology of mature oligodendrocytes in adult tracts. Dual-staining for these molecules was strongly associated with the oligodendrocyte lineage, being seen rarely in microglia and not found in astrocytes. The marked expression of these molecules in mature white matter may suggest a role in myelin maintenance and turnover.

Although our chromogenic staining demonstrated strong pIRE-1 expression in white matter tract cells, we were unable to confirm this by dual labelling. We have noted an absence of any reports containing in vivo, brain cell-specific, fluorescent dual labelling with pIRE1 and XBP1 antibodies.

In conclusion, we report for the first time significant upregulation of ATF6, pIRE1 and ER-associated chaperones in developing cerebellar white matter. Few studies compare induction of all three arms of the UPR simultaneously. By tracking the activation status of each arm of the UPR, we have shown varied activation of ER stress sensors. This is important in understanding the potential role of the UPR in oligodendrocytes during demanding physiological processes such as myelination of axons. It also has implications for development of treatments for demyelinating diseases, such as multiple sclerosis. Understanding which signalling pathways promote myelination could potentially help in the development of small-molecule drugs that can interfere with or augment these cascades, to provide a regenerative milieu in an otherwise pathological environment.

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The production, transport and integration of myelin components into the membrane during development is a highly coordinated and regulated process that relies heavily on the endoplasmic reticulum (ER), a sub-cellular organelle that is the principal site of membrane assembly. Ribosomes on the rough ER allow translation of proteins such as proteolipid protein (PLP) prior to correct folding, post-translational modification and eventual complexing with nascent smooth ER-synthesised lipids.

A single oligodendrocyte can ensheath multiple axons during developmental myelination and this process has been shown to occur during a short time window (estimated between 12–18 hours), after which the cell loses its myelinating capacity. It has been estimated that, in post-natal rat, these cells expand their surface area at a rate of 5–50×10^3 μm^2/cell/day during myelination (Baron and Hockstra, 2010) and, as such, a considerable burden is placed on the ER.

Acting as a site of quality-control in the cell, the ER ensures conformational fidelity of all its products via the action of a range of chaperones, co-chaperones and foldases and, when molecules fail to attain the correct tertiary structure, they are targeted for degradation via the ER-associated protein degradation pathway (ERAD). The ER can initiate a complex homeostatic mechanism known as the unfolded protein response (UPR) when maximal biosynthesis is occurring in a cell, and its ER is approaching maximal capacity. The series of signalling pathways that comprise this response generally results in a slowing of traffic through the ER and an expansion in ER function, allowing the cell to regain balance, but under prolonged ER stress can eventually lead to cell death.

This homeostatic mechanism is mediated by three transmembrane sensors in the ER; protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1). Under conditions of physiological stress or pathological conditions, the sensors will initiate cell-signalling pathways that work together to reduce the stress in the ER and ultimately the cell. PERK dimerises, auto-phosphorylates and, in turn, induces the activation of eukaryotic translation initiation factor alpha (eIF2α) by addition of a phosphoryl group. The net effect of this is a transient global arrest in translation. However, in a few select mRNAs containing short open reading frames in their 5' untranslated region, eIF2α activation leads to increased expression of molecules, such as activating transcription factor 4 (ATF4), whose target gene encodes molecules involved in amino acid metabolism, protein secretion and regulation of redox homeostasis. IRE1 also responds to ER stress by oligomerisation and auto-phosphorylation effecting the activation of an endoribonuclease domain that splices X-box binding protein 1 (XBP1) mRNA to produce a potent pro-survival transcription factor. This is to be expected since ATF6 has a known role in phospholipid biosynthesis, both in cultured oligodendrocytes and cerebellar explants produced oligodendrocyte-protective effects similar to those achieved by stimulating PERK (Wey et al., 2015) and has led to the proposition that the drug could be a potential therapeutic for MS. Yet, in spite of these insights into the role of this arm of the UPR in a pathological situation, what is of great interest to us is that they also found that neonatal myelination was completely impaired in PERK-null mice (Hussien et al., 2014). This suggests that the PERK arm is more crucial in the integrated stress response (ISR) that occurs in response to pathological tissue change, rather than normal physiological ER overload and highlights that different mechanisms may be occurring in reparative adult remyelination compared to during brain development. Of note, however, is that the IRE1 and ATF6 arms of the UPR were not examined in these studies.

Interestingly, immunohistochemical staining indicated that both ATF6 and IRE1 were activated at the P7 time-point, which is prior to the appearance of myelin, with maximal nuclear-localised ATF6 appearing at P10 and pIRE1 peaking at P14 (levels of pIRE1 being significantly higher at P14 and P17 than at the other pre- and post-myelination phases). ATF6’s appearance in the nucleus coincides with the earliest stages of myelination (just prior to appearance of myelin proteins) during which there is a massive biosynthesis of membrane occurring. This is to be expected since ATF6 has a known role in phospholipid biosynthesis, both in cultured oligodendrocytes and in spinally injured mice, and its activation has previously been shown to coincide with membrane protein expression in the absence of cell stress. However, it remains difficult to confirm the exact role of ATF6 activation in the expression of myelin since the ATF6α mouse seems to undergo normal neural development. However, this may be due to the compensatory mecha-
PERK signalling is known to result in a significant and global reduction of high protein and membrane manufacture, such as myelination, since al., 2010). Such a strategy would probably be beneficial in the instance activated and there is a notable suppression of the PERK pathway (Ma et


