Cerebellar pathology in multiple sclerosis and experimental autoimmune encephalomyelitis: current status and future directions

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ABSTRACT

Recent decades have witnessed significant progress in understanding mechanisms driving neurodegeneration and disease progression in multiple sclerosis (MS), but with a focus on the cerebrum. In contrast, there have been limited studies of cerebellar disease, despite the common occurrence of cerebellar symptoms in this disorder. These rare studies, however, highlight the early cerebellar involvement in disease development and an association between the early occurrence of cerebellar lesions and risk of worse prognosis. In parallel developments, it has become evident that far from being a region specialized in movement control, the cerebellum plays a crucial role in cognitive function, via circuitry connecting the cerebellum to association areas of the cerebrum. This complexity, coupled with challenges in imaging of the cerebellum have been major obstacles in the appreciation of the spatio-temporal evolution of cerebellar damage in MS and correlation with disability and progression. MS studies based on animal models have relied on an induced neuroinflammatory disease known as experimental autoimmune encephalomyelitis (EAE), in rodents and non-human primates (NHP). EAE has played a critical role in elucidating mechanisms underpinning tissue damage and been validated for the generation of proof-of-concept for cerebellar pathological processes relevant to MS. Additionally, rodent and NHP studies have formed the cornerstone of current knowledge of functional anatomy and cognitive processes. Here, we propose that improved insight into consequences of cerebellar damage in MS at the functional, cellular and molecular levels would be gained by more extensive characterization of EAE cerebellar pathology combined with the power of experimental paradigms in the field of cognition. Such combinatorial approaches would lead to improved potential for the development of MS sensitive markers and evaluation of candidate therapeutics.

KEYWORDS: Cerebellum, deep cerebellar nuclei, experimental autoimmune encephalomyelitis, multiple sclerosis, neuroinflammation

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The validity of animal models in multiple sclerosis research.

Multiple sclerosis (MS) is a poorly understood autoimmune disease targeting the brain, characterized by a wide range of symptoms and different patterns of progression between individuals. As a result, currently available treatments are of limited efficacy. Historically, approaches to study this condition have included:

1. Magnetic resonance imaging (MRI), which collects images of the brain and highlights regions of disease activity.
2. Studies on post-mortem brain, which allows analysis of the process of tissue destruction.
3. The use of animal models of the disease, in particular the one known as experimental autoimmune encephalomyelitis (EAE) which facilitates investigations of mechanisms (or biochemical reactions), especially at the earliest, pre-symptomatic stages.

In recent years, these approaches have independently revealed that a previously unsuspected brain region, called the cerebellum, actually plays a major role in the early disease stage. Therefore, these dispersed sources of information need to be collated and analysed in order to generate a clearer picture of the role of the cerebellum in MS early development and progression and implications for clinical intervention.

We performed and extensive literature search to summarize the current understanding of cerebellar damage in MS, from MRI and analysis of post-mortem tissues. Additionally, we compared these findings with evidence from the EAE model, to determine whether tissue destruction in EAE is representative of what is observed in MS. We found surprisingly good correlation between findings in MS and EAE, where equivalent relationships between mechanisms of damage and disease progression were found to exist between the human and animal counterpart of the disease.

We propose that these findings validate the EAE model for investigations cerebellar damage in MS and future evaluations of novel candidate therapeutic agents.

Introduction

MS is a common central nervous system (CNS) autoimmune and neurodegenerative disorder, frequently manifesting in early adulthood and exhibiting a higher prevalence in women, in a ratio of 3:1.[1,2] The cause of the disease and mechanisms underlying pathological processes remain obscure; however certain crucial factors contributing to MS susceptibility and initial development have been identified. These include a genetic predisposition, whereby genes within the HLA complex represent the strongest MS risk factor especially the HLA-DRB*1501 variant, but also over 230 non-HLA single nucleotide polymorphisms, each conferring a low MS risk.[3] Additionally, an increasing number of environmental factors are known to trigger or exacerbate MS, for example low vitamin D levels, viral infections including Epstein-Barr Virus, smoking, or early-life obesity.[4,5] Neither the genetic, nor the environmental component exerts a stronger influence on the risk of developing MS. Rather, the disease manifests due to interactions between environmental factors and innate and adaptive immunity which are major pathways regulated by MS risk alleles.[5]

MS is characterized by focal inflammatory lesions (or plaques) occurring in any CNS region, resulting in widely varying symptoms between affected individuals. The disease course falls into different sub-types (or phenotypes). Relapsing-remitting MS (RRMS) is the most common phenotype including over 80% of total MS cases. It is typified by intermittent periods of neurological deficits associated with white matter (WM) lesions identifiable by conventional magnetic resonance imaging (MRI), and separated by periods of remission.[6] However, concurrent with WM inflammation, is an MRI-silent cumulative neuro/axonal death and concomitant global brain volume loss.[7] In over 50% of RRMS cases, this neurodegeneration eventually reaches a threshold when neurologic reserve is depleted and the disease transitions to secondary progressive MS (SPMS),[8,9] where affected individuals progress relentlessly. A less common phenotype is primary progressive MS (PPMS), comprising 10-15% of MS cases, that presents as a continuous functional decline similarly to SPMS, without the initial RR phase.[10] Other rarer forms include paediatric MS manifesting prior to age 16,[11] or the aggressive Marburg form,[12] or benign MS, which exhibits mild symptoms and long remissions.[13] Much debate has taken place over how fundamentally distinct these subtypes are and whether MS should be more accurately viewed as a syndrome, whereby disease phenotypes are the manifestation of different pathophysiological pathways.[14,15] On the other hand, when comparing disease parameters between SPMS and PPMS, the literature reveals more similarities than differences in lesion pathology, clinical disease and rate of progression, suggesting a common driver of disease.[7,16] Therefore, whether MS forms are distinct diseases, or clinical variants of a single disorder remains an open question.

A major hurdle in the development of effective therapies has been the limited understanding of pathological processes underlying primary neurodegeneration. Current disease-modifying treatments effectively temper the inflammatory component of the pathology and consequently, attenuate the frequency and severity of relapses. However, they do not directly address neurodegeneration and lack capacity to prevent accumulation of neuronal damage/loss and progression.[17] On the other hand, it is fair to say that the last two decades have witnessed major breakthroughs in the elucidation of neurodegenerative mechanisms in MS, but these developments have resulted from an emphasis on the cerebrum. Few studies have focused on the cerebellum, which is inconsistent with the common occurrence of symptoms attributable to cerebellar dysfunction[18] and with the current appreciation of cerebro-cerebellar connectivity and participation of the cerebellum in non-motor cognitive functions.[19] Additionally, seminal studies of cerebellar pathology[20,21] have revealed early, significant grey matter (GM) damage in this region. In separate developments, imaging approaches based on experimental autoimmune encephalomyelitis (EAE), a widely used MS model, have demonstrated correlations between cerebellar GM atrophy and disease progression.[22] In this review we summarize evidence relating to EAE cerebellar pathology and highlight the relevance of these data to the human counterpart of the disease. Novel insights gained from these findings have significant implications for future investigations of MS development, progression and symptomology and potential avenues for treatment.

Current understanding of multiple sclerosis pathology.

Prior to a discussion of cerebellar pathology, an overview of current understanding MS pathophysiology and its underlying mechanisms, is required. The bulk of this knowledge has been derived from studies of the brain and to a lesser extent, the spinal cord.

The elucidation of MS pathology presents multiple challenges. First, diagnosis and patient monitoring rely heavily on conventional MRI approaches. However, conventional MRI lacks sensitivity to certain types of damage, for example GM lesions and diffuse damage in normal appearing white matter (NAWM) and in addition, does not correlate with overall disability.[23] Additionally, there has been much reliance in post-mortem (pm) tissue analysis which suffers from oversampling of end stage pathology, resulting in loss of information on early processes susceptible to alterations over disease evolution. Furthermore, pathological investigations can be biased towards regions of macroscopic damage, with diffuse and subtle damage being overlooked, for example in NAWM and cortical lesions. Nonetheless, the development of advanced MRI modalities has resulted in increased sensitivity for the detection and

characterization of disease activity. Similarly, improved protocols for post-mortem tissue preservation and the advent of brain banks have facilitated the application of a wider range of immunochemical approaches and state-of-the-art molecular techniques. Altogether, an improved spatio-temporal vision of the evolution of MS pathology and of its complexity, has emerged.

The most salient feature of MS pathology is the WM plaque[24,25] in the brain and spinal cord,[26] which forms around post capillary venules as a result of blood brain barrier (BBB) loss of function. Lesions contain T cells, (especially $CD8^+$), B cells and macrophages containing myelin debris, associated with axonal injury and prominent astrogliosis and microglial reactivity. While these active lesions predominate in early disease, other lesion types including chronic active plaques (characterized by low demyelinating activity at the lesion edge and less extensive BBB damage), inactive lesions (exhibiting few macrophage/microglia or T cells), mixed active/inactive and shadow plaques (sharply demarcated areas with reduced myelin density and disproportionately thin myelin sheaths) are also evident. In progressive stages, active focal lesions become rarer, while increasing numbers of slowly expanding lesions, which can eventually merge, are more often observed.

The characterization of pathogenic processes within active WM lesions has clinical implications in terms of diagnosis, prediction of disease course and therapeutic approach; however, apparently contradictory evidence has been presented. One line of investigation,[27] using biopsies from early disease (taken for diagnostic purposes) and autopsies, identified four distinct pathological patterns, whereby each pattern was homogeneous across lesions from any given case but heterogeneous between cases. MRI lesion characteristics reflect specific morphological features of histologically classified immunopathological patterns,[28] implying that pathological heterogeneity represents differential disease mechanisms. A contrasting study,[29] examining lesions from patients dying during or shortly after onset of a relapse identified severe oligodendrocyte apoptosis and microglial reactivity associated with demyelination as the earliest pathological changes. The concurrent absence of infiltrating lymphocytes suggests autoimmunity to be secondary to oligodendrocyte loss. Interestingly, lesion heterogeneity within single cases was identified leading to the conclusion that pathological heterogeneity reflects lesion stage.[29,30] In summary therefore, the determination of events underlying the development of WM lesions remains work in progress.

In GM, active lesions are observed from early stage, principally in deep grey matter nuclei, including the basal ganglia, thalamus, hypothalamus, as well as in spinal cord GM.[7] They are characterized by perivascular inflammation, microglial reactivity, demyelination and neuronal loss. However, more recently attention has focused on cortical lesions, due to their severity and potential clinical significance. Cortical lesions are poorly detectable by MRI, with only 10-15% being identified, while damage identified by pm examination extends over 20-30%,

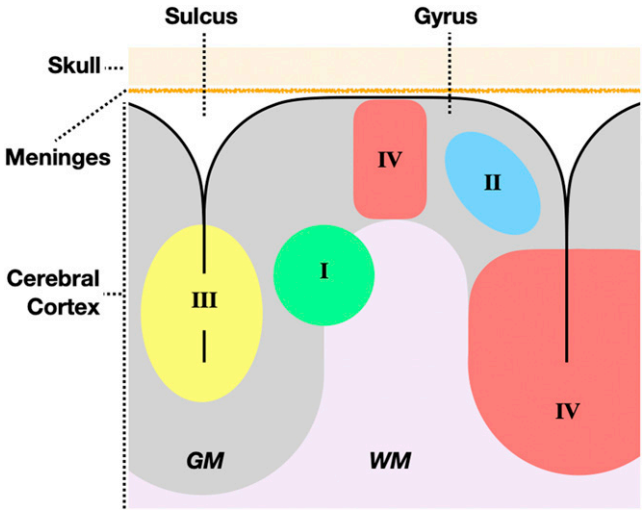

Figure 1. Grey matter lesion classification. Type I (green) occur at the boundary of WM and GM and extend into both. Type II (blue) are purely intracortical and do not reach either pial surface or WM boundary. Type III (yellow) lesions present as extensive subpial demyelination extending a short way into the cortical surface. Type IV (red) span the entire GM cortex reaching but not extending into the subcortical WM (30).

or even above 70% of the cortex in extreme cases.[31-33] These lesions are associated with severe meningeal (rather than perivascular) inflammation, rich in aggregates of T and B cells and plasma cells, resembling tertiary lymphatic follicles in the most severe forms. Lesions themselves, however, are characterized by limited BBB breakdown, paucity of T cells and macrophage infiltration relative to WM lesions, but abundant demyelination associated with extensive axonal transections, neuronal apoptosis and reduced neuronal and synaptic density.[32] Thus, while lymphocytes and plasma cells are confined to the meninges, reactive microglia accumulate at sites of active demyelination; therefore it is proposed that cortical neurodegeneration is driven by soluble factors from the meninges inducing demyelination and neurodegeneration by provoking microglial reactivity.[7] Our studies based on EAE implicate platelets/platelet-derived factors as candidate substrates of neurodegeneration.[34,35] Cortical GM lesions also display heterogeneity and belong to four sub-types:[31,32,36,37] Type I consists of leukocortical lesions at the cortical GM/WM boundary; type II lesions are small, purely intracortical and surrounding blood vessels; types III/IV present as extensive demyelination occurring in a ribbon-like span across including entire or multiple gyri, but differing in terms of cortical depth with type III extending to layers 3-4 and type IV across the entire cortex (Figure 1). Examination of brain biopsies collected within days to weeks of presentation show that these lesions are already occurring in early disease and are initially highly inflammatory, thereby demonstrating that early cortical lesions differ substantially from those at end-stage.[38] Of note, is the absence of significant differences between SP- and PPMS in numbers and size of cortical lesions,[7] again supporting the

notion that these phenotypes result from common pathogenic processes. Thus, mechanisms underpinning cortical damage remain unresolved overall, but their functional consequences may be related to cognitive and executive dysfunctions and neuropsychiatric symptoms such as dementia and seizures in MS patients.[33]

Progressive disease also exhibits WM and GM diffuse damage, consisting of $CD8^+$ T cell infiltration associated with axonal injury, secondary demyelination and astrocytic and microglial reactivity. Diffuse demyelination is contributed to by secondary Wallerian degeneration resulting from axonal and neuronal damage in focal lesions in WM and GM, but other factors for example, meningeal inflammation and microglial reactivity. Finally, there is considerable evidence for the critical involvement of B cell subsets in MS development and progression, highlighted by the recent success of B cell depleting therapies.[39] B cell involvement has long been recognized from evidence of cerebrospinal fluid (CSF) Ig, as well as Ig and complement deposition in areas of active demyelination, but can potentially contribute to pathogenesis via antibody-independent functions, including as potent antigen presenting cells and promotion of a pro-inflammatory environment by the generation of cytokines. B cells can also exhibit anti-inflammatory effects, therefore more needs to be known about particular B cell subsets contributing to disease development and their clinical relevance to MS progression.[40]

Altogether, these combined processes bring about irreversible demyelination and neuro-axonal loss over time, resulting in GM and whole brain atrophy. The consensus is that global brain atrophy is the strongest correlate to overall progression.[7,8,41,42]

Cerebellar disease in multiple sclerosis

Structure and function of the cerebellum

The cerebellum resides in the posterior cranial fossa, behind the 4^{th} ventricle, the pons and medulla oblongata. It is a furrowed structure densely packed with neurons, with folds (or folia), giving rise to gyri (or ridges) and sulci (grooves). The significance of this structure is evidenced by the fact that it contains over 50% of total brain neurons[19,43] and that its rate of size change over evolution in humans strongly correlates with that of the neocortex.[44] It consists of an inner WM core enveloped by a cortex, made up of three layers:[45,46] an internal granular layer, a Purkinje cell layer and an external molecular layer (Figure 2). The granular layer is named after the small, granular in appearance and densely packed excitatory neurons that constitute the bulk of cells in this region. These neurons have a simple architecture with a small amount of cytoplasm, 3-4 dendrites and one, thin axon which connects to Purkinje cells. By contrast, Purkinje neurons which are aligned in an orderly layer are large and endowed with complex dendritic arborization and numerous dendritic spines that project into the molecular layer. They possess long axons which traverse the granular layer, where they become myelinated, terminating into cerebellar and brainstem nuclei. They are the only output neurons of the cerebellar cortex and therefore, central to cerebellar cortical information processing. The molecular layer is a cell-poor region containing Purkinje dendritic arbors, excitatory projections from the granular neurons that interact with these arbors, as well as interneurons (stellate and basket cells). Although the cerebellar cortex is overall poorly myelinated, prominent myelin bands lie above and below Purkinje cells (infra and supraganglionic myelin sheaths) and moderate myelination is found within the granular layer.

Within the WM are the deep cerebellar nuclei (DCN) (Figure 2), namely the fastigial, interposed, and dentate nuclei. The fastigial nucleus is the oldest phylogenetically, the smallest and medially located. Its functions include axial and proximal motor control and physiological functions including feeding, cardiovascular, respiratory, as well as emotional regulation which are highly evolutionarily conserved.[47] The interposed nucleus is subdivided into the globose and emboliform nuclei and responsible for co-ordinating agonist/antagonist muscle pairs; therefore damage in this region results in tremors. It also exerts cognitive functions such as acquisition and retention of classically conditioned behaviours.[48] The dentate nucleus is the largest and most laterally located and responsible for planning, initiation and control of voluntary movement and damage in this region results in ataxia. It also has non-motor functions in cognition and sensory processing.[49] As the Purkinje neurons are for the cerebellar cortex, DCN neurons are responsible for almost all output of the cerebellum to other brain areas.

The cerebellum connects to the brainstem via three paired white matter tracts, namely the superior, middle and inferior cerebellar peduncles. The output of the superior peduncle is almost exclusively efferent, while the middle peduncle is an entirely afferent pathway and the inferior peduncle contains both afferent and efferent pathways.[45] Projections into the cerebellum pass through the peduncles along mossy fibres originating from brainstem nuclei and the spinal cord and activating granular cells, or climbing fibres from the inferior olive activating Purkinje cells. Thus, information travels through the cerebellar WM to the cortex, is processed in the molecular layer and sent from the cortex to the DCN via the Purkinje layer before leaving the cerebellum via the superior and inferior peduncles.

Historically, the cerebellum was regarded as a structure essentially associated with motor control, or motor impairments such as tremor, gait ataxia, or oculomotor disturbance.[50-52] However, anatomical, neurophysiological and neuroimaging studies have identified cerebellar regions connected to cerebral association areas, resulting in an appreciation of the role of the cerebellum in processing cognitive information.[53-55] Therefore, it is not surprising that cerebellar deficits are a common feature of MS and other symptoms including impaired executive function, memory, verbal fluency or emotional regulation are also recognized as consequences of cerebellar dysfunction.[56-58]

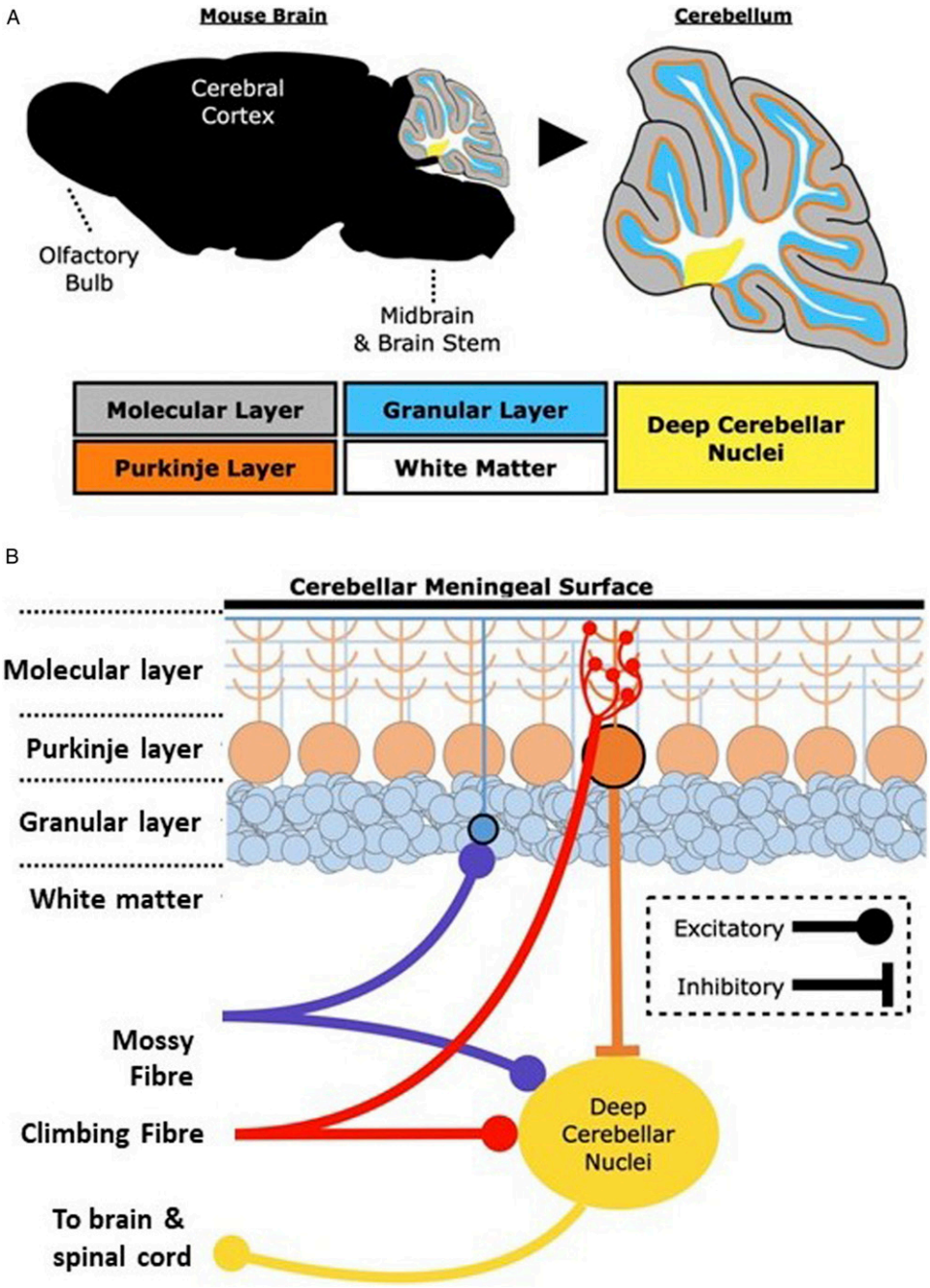

Figure 2. Cerebellar anatomy and circuitry. (A). Sagittal view through an adult mouse cerebellum. Immediately beneath the meningeal surface is the molecular layer (grey), the bulk of which is made up of the expansive dendritic arbors of the Purkinje neurons located immediately beneath, within the Purkinje layer (orange). The innermost layer of the cerebellar cortex is the granular layer (blue). Beneath the cerebellar cortex lies the white matter which in turn surrounds the deep cerebellar nuclei (yellow). (B). A simplified representation of the cerebellar circuitry. Inputs into the cerebellum enter the super, middle or inferior peduncles (not pictured). Climbing fibres (from the inferior olive) pass through the WM and connect to granular neurons in the granular layer, which then relay information through to Purkinje neurons via their dendritic arbors in the molecular layer. All other inputs into the cerebellum are received via mossy fibres, so named for their extensive connections to a small number of Purkinje neurons (usually less than 10). Alternatively, both climbing and mossy fibres also project directly to the deep cerebellar nuclei. Regardless of the input (climbing or mossy fibre), all cerebellar cortical out is derived from the Purkinje neurons, almost all of which project to one of the four deep cerebellar nuclei (fastigial, interposed (emboliform and globose) and dentate) which in turn project to targets throughout the brain and spinal cord.

The prevalence of cerebellar symptoms in MS is uncertain, because they involve other CNS regions, but cerebellar signs are estimated to constitute the predominant clinical manifestation in 11-13% of people with MS.[18]

Also, of particular relevance to MS are differences between men and women in the brain architecture and circuitry, where for example, men have a greater cerebellar volume than women. Furthermore, distinct differences in the connectome in both the

cerebral hemispheres and cerebellum have been demonstrated, with greater within-hemispheric connectivity in males, but greater between-hemispheric connectivity in females in the cerebellum.[59,60] This implies that sex-related differences would impact on symptoms and management of the disease. Finally, an additional incentive for increased focus on cerebellar involvement in MS stems from genome-wide associated studies (GWAS),[3,61] which have consistently identified variants of moderate to large effect sizes that meet genome-wide significance thresholds, but also multiple genetic loci of small effect sizes. This latter group appears to have no effect on disease onset, but to play a major role on clinical outcomes via their associations with biochemical processes and pathways determining disease severity and clinical course. A recent study identified an overrepresentation of genes expressed in CNS compartments generally, and specifically in the cerebellum. These involved mitochondrial function, synaptic plasticity, cellular senescence, calcium and g-protein receptor signalling pathways.[62]

Cerebellar pathology in multiple sclerosis

Cerebellar white matter and cortical pathology. The cerebellum is a major site of predilection for lesion development in MS, involving both WM and GM. WM lesions are widespread throughout the structure, including in the cerebellar peduncles and characterized by inflammatory infiltration, demyelination and severe microglial activation, similarly to brain and spinal cord lesions.[20,46] Both active lesions and inactive lesions are observed concurrently, but whether pathologically distinct lesions subtypes occur has not been documented. Additionally, there is widespread pathology in the cerebellar cortex in all MS subtypes,[21,63] but only two lesion types have been documented. These include leukocortical lesions, which represent extensions of WM lesions in the folia abutting the adjacent cortical layer and sub-pial lesions which are more extensive. The latter are characterized by near total demyelination occurring in a ribbon-like span across multiple folds, involving all cortical layers. Relative preservation of axons is observed, except for the uncommon observation of axonal swelling and terminal bulbs, which are features of degenerating axons.[31] Cerebellar cortical lesions are typified by paucity of T and B cells, but pronounced microglial reactivity. Also documented is significant loss of Purkinje neurons. These pathological features are associated with meningeal inflammation rich in $CD20^+$ B cells and IgA^+/IgG^+/IgM^+ plasma cells, $CD4^+$ and $CD8^+$ T lymphocytes and $CD68^+$ monocytes/macrophages, which in SPMS cases, correlate with the occurrence of lymphoid structures in the cerebrum.[64] The demyelination pattern is suggestive of a toxic factor diffusing from the deep meningeal infolding, with myelin loss initiated at the outermost areas followed by supra/infra-Purkinje layer myelin, granular layer then white matter.[20]

Cerebellar cortical demyelination also strongly correlates with that seen in the cerebral cortex,[8,33] but although variable, cerebellar demyelination exceeds that of the cerebral cortex: it was found that in PPMS and SPMS patients on average 38.7% of the cerebellar cortex was demyelinated, with extreme cases reaching up to 92%. However, in RRMS this amounted to around 7.5%, suggesting that cerebellar cortical demyelination is either a feature of progressive disease (in contrast to the cerebrum), or a consequence of cumulative pathology. On the other hand, no correlation between cerebellar GM and WM demyelination was found and GM demyelination could be seen in near absence of WM lesions.

Deep cerebellar nuclei damage in multiple sclerosis. Studies of the DCN in MS are rare. One report of 16 pm MS cases and 8 healthy controls,[65] revealed demyelination in 7 MS cases and significantly reduced synapse numbers in non-demyelinated and demyelinated dentate nuclei compared to controls, more pronounced where demyelination was present. Significant neuronal loss was demonstrated, sometimes associated with shrunken and hyperchromatic neuronal soma. Astrocytic reactivity was evident and strikingly, glial processes were in close apposition with dentate synapses. Ultrastructural analysis of one MS case suggested synaptic degradation resulting from both glia-mediated separation of synaptic boutons from the neuronal soma and a neuron-autonomous mechanism typified by autophagosomes containing synaptic components.

Functional consequences of cerebellar damage.

MRI is the gold standard technique to support a clinical diagnosis of MS, but this technology is being used with increasing sophistication towards the development of sensitive markers for early detection, patient monitoring, prognosis and evaluation of candidate therapeutics. Imaging of the cerebellum is uniquely challenging, due to the difficulty regarding correct segmentation of the thin sulci and gyri and differentiation between cerebellar tissue and adjacent structures. Generally, conventional MRI approaches provide morphological evaluations, while advanced MRI modalities allow more specific interrogation of microstructural and functional alterations.[66] The emerging evidence is that cerebellar lesions appear early, with about 20.5% of patients exhibiting at least one cerebellar lesion at the clinically isolated syndrome stage ([CIS] defined as the first demyelinating event prior to definite diagnosis), while 49% of patients with clinically definitive MS (CDMS) exhibit cerebellar lesion(s). The detection of at least one lesion at the CIS stage is associated with a high risk of conversion to CDMS and worse prognosis.[66] Both WM and GM are affected: a study of twenty-eight MS patients and sixteen healthy controls[67] demonstrated lesions in 11/14 RRMS and 13/14 SPMS cases, typically in peduncles and hilar regions of the DCN (predominantly associated with RRMS), as well as leukocortical and pure cortical lesions (predominantly in SPMS) which correlated with cognitive disability. In this study, combined PET/MRI, using high specificity radiotracer for reactive microglia identified widespread inflammation,

including in normal appearing tissue. Alongside lesion formation, cerebellar volume loss also occurs and the overall data show reduction of total cerebellar volume compared to healthy controls, more pronounced in progressive patients, but discrepancies are observed between data sets with respect to changes in GM and WM and between MS phenotypes probably because of small patient cohorts in some studies, or difficulty of distinguishing between WM and GM in thin folia. One investigation[68] identified cerebellar cortical atrophy and lesions in all MS subtypes, already present from the CIS stage. While significant WM atrophy was not a feature of early disease, low correlation between cerebellar cortical volume loss and WM lesion load was found. By contrast, a separate study[69] reported reduced WM volumes at the CIS stage. A third study[70] reported borderline GM volume reduction is RRMS patients, but significant reduction in SPMS patients, while reduced WM volume was only seen is SPMS. In this and a further study,[71] cerebellar GM volume loss was found to be a significant predictor of cerebellar dysfunction thereby implying that cerebellar GM atrophy is related to progressive disability.

Structural imaging techniques have identified early microstructural changes in WM especially in the peduncles, even in absence of focal lesions,[72] which correlate with motor/ambulatory difficulties.[73] Initial investigations demonstrated associations between damage to the superior peduncle and upper limb dysfunction, or to the superior and middle peduncles and reduction in cerebellar GM volume with impaired postural control.[74-76] More recent reports[76] demonstrated involvement of all three cerebellar peduncles, thereby implicating both afferent and efferent pathways. Structural imaging studies correlate with magnetic resonance spectroscopy for estimations of neuroaxonal markers, particularly N-acetyl aspartate, which revealed cerebellar axonal loss associated with ataxia.[74] MS is also characterized by reorganization of functional connectivity identified by fMRI, which measures blood oxygenation levels at rest or during motor, or cognitive tasks. This modality can quantify relationships between structural damage in specific structure/sub-region, loss of connectivity, complex processes and MS phenotypes. As examples, fMRI has revealed a link between lesion load in cerebellar peduncles and impaired functional integration in the cerebellar cortex, as well as altered functional connectivity between the cerebellum and pre-motor cortex.[77,78] Combined fMRI and posturography studies showed that worse posturometric values correlated with altered cerebro-cerebellar connectivity, especially reduced connectivity between the dentate nucleus and caudate nuclei and thalamus, but increased connectivity with other cerebellar structures.[79] Overall, the posterior cerebellum is emerging as a significant region implicated in cognitive processing, whereby reduced posterior volume is predictive of worse cognitive performance. Two studies of paediatric-onset MS revealed posterior cerebellar reduction correlating with cognitive function, especially impaired information processing speed and vocabulary, thereby identifying early cerebellar damage, together with a role for cerebellar pathology in paediatric-onset MS.[66] The issue is whether these abnormalities result from primary cerebellar dysfunction, compensatory, or maladaptive changes.

Therefore, in summary, cerebellar and cerebral hemisphere MS pathology exhibit multiple common features in terms of hallmarks of WM and GM damage (Table 1) and also, the relationship between cerebellar cortical volumetric changes and disease progression. The implication of early cerebellar involvement is highlighted by demonstration of a correlation between presence of lesions at the CIS stage and risk of conversion to CDMS. Most importantly, however, the identification of functional consequences of cerebellar damage on cognitive processes, which is in accordance with the new appreciation of cerebellar functions beyond movement control, is of high significance for diagnosis and treatment strategy.

With these advanced MRI modalities offering increased sensitivity and spatial information in the detection of disease activity and severity, it may be possible in the near future to address the relationship(s) between certain confounding aspects of MS, such as sex-related differences or ethnicity and cerebellar involvement. Firstly, sex-related differences in cerebellar pathology are still poorly explored in MS, which is at odds with the level of interest of these differences in other aspects of the disease. However, we have uncovered one very recent MRI study exploring the effect of sex on upper extremity function (with the 9-hole peg test) and potential anatomical and functional substrates,[80] where sex-related differences were identified in the cerebellar network, with a stronger negative correlation in the left cerebellum in men compared to women. Although these correlations appear to mirror sex-related anatomical differences, the limitation of these studies is the moderate sample size (n = 50-70 per group), which also precludes further determination of relationships with distinct MS phenotypes. Second, with respect to ethnicity, the differences between prevalence, risk factors and disease severity between racial and ethnic groups is well established,[81] with lower prevalence in Asia compared to western countries, but lower prevalence and more aggressive progression in African-Americans than Caucasian Americans. A study of 66 Japanese MS patients[82] revealed cerebellar involvement in 6.4% of cases (therefore, significantly lower than in the western population),[18] with lesions identified mainly in the peduncles. By contrast, a separate study showed significantly higher cerebellar atrophy in African-Americans relative to Caucasian Americans, mainly involving posterior lobules and cerebellar atrophy being the best predictor of MRI metrics between these racial groups.[83] This study is in agreement with other data revealing higher incidence of cerebellar dysfunction and more rapid accumulation of disabilities in African-Americans.[84] Studying racial differences in cerebellar pathology is important, because of the significance of MRI evidence of early cerebellar lesions:[66] they may be related to fundamental differences in the clinical phenotype and natural history of MS between racial groups, which has implications on evaluating prognosis and response to disease modifying therapies.

Table 1. Comparison between hallmarks of cerebrum and cerebellum pathology. Pathological hallmarks of lesions in WM, deep GM nuclei and cortex in the cerebrum and cerebellum and are listed. WM lesions exhibit similarities between the two regions, although these lesions have been less extensively studied in the cerebellum. Lesions in deep GM nuclei have been characterized in the cerebrum only. Cortical lesions in the cerebrum and cerebellum exhibit similar hallmarks; however they appear more extensive in the cerebellum than in the cerebrum by disease end-point and there is a correlation between cerebellar lesions in the CIS stage and prognosis.

PARAMETERS	CEREBRUM	CEREBELLUM
WM lesions cellular profiles	Associated with peri-vascular inflammation. Plaques contain T cells (predominantly $CD8^+$), B cells and macrophages containing myelin debris. Widespread across the cerebrum	Associated with peri-vascular inflammation. Plaques contain T cells (predominantly $CD8^+$), B cells and macrophages containing myelin debris. Widespread across the cerebellum, including cerebellar peduncles
	At least four distinct sub-types identified, based on pathological hallmarks	No study reporting potential occurrence of pathological sub-types
Astroglial and microglial reactivities	Prominent	Prominent
Axonal damage and loss	Very severe	Very severe
Deep GM nuclei	Observed from early stage. Characterized by perivascular inflammation, microglial reactivity, demyelination and neuronal loss	Insufficient data
Cortical lesions Cellular profiles	Associated with peri-vascular inflammation. Characterized by limited BBB loss of function, paucity of T cell and macrophage infiltration relative to WM lesions. Poorly detectable by MRI.	Associated with peri-vascular inflammation. Characterized by limited BBB loss of function, paucity of T, B cells and plasma cells and macrophage infiltration relative to WM lesions. Poorly detectable by MRI.
	At least four distinct sub-types, based on pathological hallmarks identified	Two of the 4 sub-types identified in the cerebrum observed
Microglial reactivity	Prominent	Prominent
Neuronal loss	Extensive axonal transections, neuronal apoptosis and reduced neuronal and synaptic density	Extensive axonal transections, neuronal apoptosis and reduced neuronal and synaptic density. Significant loss of purkinje neurons
Cortical demyelination	Already severe in early disease. At post-mortem, extends over 20-30% of cortex, or even up to 70% in some cases	At post-mortem, extends on average to 38.7% of cortex, or up to 92% in some cases. Unclear whether it begins early, or is associated with progressive disease
Correlation with prognosis		Cerebellar lesions appear early. At least one lesion at CIS stage associated with high risk of conversion to CDMS.
Correlation with progression	Global brain atrophy is the strongest correlate to overall progression	Correlation between cerebellar GM volume loss and disease progression

Cerebellar disease in multiple sclerosis animal models.

Multiple sclerosis experimental models

There is no accurate MS model. Instead, several experimental paradigms have been created to investigate different MS facets. Commonly used ones include:

1. *Theiler's murine encephalomyelitis virus*-mediated CNS inflammation,[85] which attempts to replicate the potential viral aetiology of MS. Disease severity and course vary between mouse strains. The pathology is characterized by lymphocytic infiltration, neuronal and oligodendrocyte apoptosis and demyelination, which is predominantly periventricular and along the spinal cord.
2. *Toxin-induced demyelination* (cuprizone[86] and lysophosphatidylcholine)[87] models, which represent reproducible models for inducing and examining demyelinating/remyelinating events. Upon exposure to the toxin, mice exhibit extensive reactive gliosis, activation of microglia, oligodendrocyte apoptosis and demyelination. Since cuprizone is administered orally (over 5-6 weeks), demyelination becomes widespread throughout the CNS including WM, cerebral and cerebellar cortical GM and cerebellar peduncles. It is, however, most reliably observed in the corpus callosum, which is the preferred region examined. Lysophosphatidylcholine is delivered via injection into the spinal cord; therefore demyelination remains localized to the site of injection. Spontaneous and extensive (but usually incomplete) remyelination is observed upon toxin withdrawal.
3. *EAE*,[88,89] which remains the preferred model and is induced by immunization of rodents and non-human

Table 2. Properties of murine and marmoset EAE. The most salient differences between murine and marmoset EAE, impacting choice of species and experimental design are listed. In general, the relatively low cost, less demanding level of care, availability of genetically modified lines and potential for robust statistical analyses, but limited translatability make murine EAE more appropriate for proof-of-concept studies. On the other hand, the closer evolutionary relationship of marmosets to humans and more representative clinical, immunological and pathological characteristics, as well as potential for longitudinal studies make marmoset EAE more relevant to MS, despite costs and high level of care required.

PARAMETERS	MURINE EAE	MARMOSET EAE
Ethical considerations	Lower cognitive ability than NHP. Relatively less enrichment required	High cognitive ability. Higher standards of psychological well-being and enrichment required. Highly debilitated animals have special husbandry needs
		Use is justified only when question cannot be addressed using evolutionary lower species
Housing and costs	Relatively low cost compared with marmoset EAE. Mice are group-housed and require relatively less veterinary and animal care staff attention	High cost of animals. NHP require more space and veterinarian and animal care staff attention
	Housed in specific pathogen free (SPF) environments; immune system develops in the presence of minimal environmental influence	Housed in conventional environment; immune system shaped in the presence of exposure to wide range of microorganisms
Genetic heterogeneity	None. Inbred lines used	Maintained by out breeding and reflecting human heterogeneity
Disease profile	Highly predictable. Determined by strain/encephalitogenic protein or peptide. Disease onset synchronous	Unpredictable; mostly chronic-progressive profile, can also exhibit chronic-relapsing EAE. Disease onset variable
Disease incidence	Ranges from ≥66% to ~98%, depending on induction protocol and EAE variant	~100%
Lesion topography	Predominantly spinal cord lesions	Lesion topography mirrors that of MS. Cortical lesions identified
Genetic manipulation	Commonly performed. Large numbers of genetically modified strains available	Not available
Experimental design	Extensive range of mouse-specific reagents and established protocols	Very low availability of species-specific reagents
Data analysis	Large cohorts can be used facilitating robust statistical analyses	Statistical analyses difficult to perform, due to low numbers used in experimentation
	Sampling of CNS and other tissues possible at multiple points along disease course, due to ethically accepted killing of mice	Blood but not CNS tissue sampling possible
Relevance to MS	Limited. More suitable to provide proof-of-concept for mechanisms underpinning pathophysiology	Clinical and pathological features, and immunological mechanisms more representative of MS than rodent EAE due to closer evolutionary relationship
Translatability	Limited. Inconsistent in predicting the efficacy of candidate MS therapeutics	In progress. Validated for 'reverse translation' studies, namely analysis of basis of failure or success of defined candidate therapeutics.[83,93]

primates (NHP), with CNS proteins or peptides, in the presence of adjuvants (Table 2). Murine EAE exists as multiple variants, generated from defined mouse strain/neuroantigen combinations, resulting in the expression of differing disease profiles, including chronic-relapsing, chronic-progressive, or monophasic EAE. The variant produced with peptide 35-55 of the CNS-specific myelin component denoted myelin oligodendrocyte glycoprotein (MOG_{35-55}) in the C57BL/6 mouse strain has been favoured, partly due to the prominent place of this mouse strain in genetic modification strategies. Murine EAE exhibits ambulatory difficulties, balance problems, bladder and bowel incontinence and pre-onset neuropsychological deficits.[90,91] Florid WM lesions are evident, characterized by severe meningeal and perivenous inflammation and lesions share common histopathological hallmarks with pattern II of the WM lesion classification. Paucity of GM lesions is observed in most EAE variants, but the development of EAE with GM pathology was recently demonstrated.[92] For example, one model was generated by stereotactic injection of pro-inflammatory mediators and subsequent subclinical MOG immunization. This resulted in early demyelinating lesions in the cerebral cortex with hallmarks reminiscent of cortical MS lesions,[38] which resolved rapidly. This rapid resolution may partly explain the observation of highly inflammatory lesions in early disease,[38] vs chronic demyelinating lesions in post-mortem tissue. The potential for the generation of novel variants, each recapitulating a defined facet of MS

emphasizes the versatility of the model and the basis for its popularity.

NHP EAE has been successfully generated in macaques and marmosets, with the marmoset as preferred host.[94,95] It is induced with whole myelin, the soluble moiety (amino acids 1-124) of MOG, or MOG peptide 34-56. Importantly, breeding can be controlled so as to generate outbred animals. Clinical and pathological features and immunological mechanisms underpinning marmoset EAE are more representative of MS than rodent EAE, due to the closer phylogenetic relationship between NHP and humans.[96-98] Marmoset EAE exhibits chronic-relapsing, or PP clinical profiles. WM lesions also exhibit pattern II hallmarks, suggesting an association between this pattern and the use of MOG as antigen. They include active, inactive and confluent demyelinating plaques[99] while lesion topography mirrors that of MS, including occurrence in the cerebellar WM and peduncles.[100] Cortical GM lesions are found in the cerebrum, including leukocortical, intracortical and sub-pial sub-types, with hallmarks resembling those of MS,[96,97,101] while MRI shows reduced cortical volume.[96]

Cerebellar pathology and dysfunction in experimental autoimmune encephalomyelitis

Despite the wealth of data on marmoset EAE pathology and MRI, studies have been limited to the cerebrum. Murine cerebellar pathology has received more attention and has been identified in several variants.[102-105] Of significance, is the demonstration of cerebellar atrophy and its pathological correlates, in some elegant studies.[93,106,107] First, histological examinations revealed both WM and GM inflammation by clinical onset. In WM inflammatory infiltration was characterized by perivascular lesions together with demyelination and axonal loss. In GM diffuse inflammatory infiltration in all cortical layers was observed, associated with demyelination, microglial reactivity, as well as swollen axons and axon retraction bulbs, characteristic of MS axonal injury.[31] Neuronal apoptosis was prominent in the granular and Purkinje layers, the latter appearing disorganized with altered arborization and reduced soma size. Second, ex vivo and in vivo MRI evaluations were performed with the use of methods eliminating bias resulting from large errors associated with significantly different morphology, such as disease-induced changes. Data revealed significantly decreased global cerebellar volume relative to controls, notably in cortical volume and specifically in the molecular layer, which showed an inverse correlation with disease duration; that is, the gradual reduction in cerebellar volume occurred as a function of time and was related mainly to GM volume reduction. Also, significant decrease in Purkinje cell numbers correlated with molecular layer atrophy. Finally, WM volume loss showed no correlation with whole cerebellar volume loss, thereby implying that cerebellar cortex loss is the strongest correlate for cerebellar atrophy. A separate investigation[108] over chronic long-term disease duration, examined up to 62 CNS sub-regions. This study demonstrated an increase in total brain volume at the peak of disease, relative to sham-induced and healthy controls, but significant decrease at experimental endpoint relative to the peak. When EAE mice were subdivided according to disease severity, high score mice had significantly smaller total brain volume and cerebellar volume relative to low score mice and control groups. These changes were associated with cerebellar decrease in neuronal density, demyelination and reduced axonal staining. Taken together, data show that initially inflammation causes CNS swelling, but with disease progression resolution of inflammation occurs, associated with accumulation of neurodegeneration and manifested by reduction in overall brain volume including reduced cerebellar volume.

In addition to the above histological and imaging combinatorial approaches, further evidence of cerebellar damage in EAE is available. Immunopathological approaches have revealed early demyelination of Purkinje axons and damage to Purkinje axons and soma in EAE.[109] Additionally, altered Purkinje neuron protein expression associated with abnormal firing patterns and motor symptoms have been documented. Examples include reduced expression of the metabotropic glutamate receptor mGlu1a (adult form) coupled with increased mGlu5 (developmental form) expression,[110-112] or $Na_v1.8$ channel proteins and annexin light chain, normally expressed in the peripheral nervous system.[112] Alongside cortical volume loss and Purkinje channelopathies, was a reduction in the inhibitory interneuron population also contributing to altered firing patterns,[110] while increased numbers of binucleate Purkinje neurons/bone marrow derived cells was observed, presumed to be a survival response, mitigating the effect of Purkinje cell channelopathy on electrical activity. These findings are of clinical significance because equivalent changes in MS pm tissues of Purkinje cell morphological changes,[113] neuron specific sodium channel,[114] as well as mGlu1a receptors[114] have been reported.

Altogether, these combined studies reveal early and significant cerebellar GM damage associated with altered firing patterns in EAE, with likely functional consequences to cerebellar-cerebral hemispheres communication. Also of significance is the observation of increasing cortical involvement over the disease course which correlates with cerebellar atrophy. Thus, the available data strongly support the validity of EAE as a model for cerebellar MS pathology.

A role for experimental autoimmune encephalomyelitis in understanding multiple sclerosis cerebellar damage.

Validity of experimental autoimmune encephalomyelitis as a model of multiple sclerosis.

EAE and MS are distinct diseases and the frequent failure to integrate this discrepancy in experimental design has led to controversy regarding the validity of the model.[115-117] Critiques argue that EAE is an induced disease, requiring strong

adjuvants to develop and only partially recapitulates MS. Rodent EAE relies on different variants requiring inbred strains to investigate clinical, immunological and pathological MS facets, thereby losing the genetic heterogeneity inherent in human populations. The evolutionary distance between hosts and humans has implications on translatability, in terms of ratio of cortical layer thickness to total brain volume, which impacts on interpretation of imaging studies, and additionally, in the complexity of infolding which has relevance to cortical demyelination. Thus, it is hypothesized that the convoluted human cortex is associated with slower blood flow in the sulci, facilitating diffusion of myelinotoxic components into adjacent GM and that an equivalent mechanism is unlikely in murine brains. Finally, the model has been inconsistent in predicting the efficacy of candidate therapeutics.[118-120] On the other hand, due to the potential for genetic manipulation of immune and CNS elements, rodent EAE has provided valuable insights into immune-mediated injury, including mechanisms of BBB loss of function, differentiation between $CD4^+$ and $CD8^+$ subsets, the role of the Th17 subset and cytokine/chemokine networks. NHP EAE has the advantages of greater similarity with its human counterpart and potential for performing longitudinal studies. Furthermore, recent work described above has highlighted the similar hallmarks between murine and NHP WM lesions and type II human lesions and demonstrated the feasibility and potential of murine cerebellar imaging, by identification of cerebellar volume loss and its correlates.[93,107,108] Overall, no experimental paradigm other than EAE demonstrates pathological features reminiscent of MS, or the capacity to address the autoimmune response, nor allows repair and neuroprotective strategies to be explored.[115,116,118]

It is also important to remember the significant contribution of murine and NHP models in establishing the foundations of current understanding of functional anatomy, CNS circuitry and behavior, by serving as principal experimental tools decades ahead of the advent of advanced imaging. These studies remain highly relevant to the understanding of MS symptomology:

1. *The cerebro-cerebellar system anatomy and functionality*. Comparative neuroanatomy has established the general functional conservation in the mammalian brain system. Advances in expression profiling approaches have revealed that this conservation extends to inter-species gene expression homology at the levels of both individual genes and gene patterns.[121] Thus, comparative gene expression between homologous brain regions across mouse, NHP and humans reveals that regionally enriched expression in one species is replicated in homologous regions of the other species for single genes, including in the cerebellum. Similar conservation of expression ratios in gene patterns is observed.[121]
2. *Cognitive function*. There is consensus that non-motor cerebellar functions in NHP, such as sensory processing, discrimination learning, spatial learning, motivation and emotion parallel those of humans;[122] hence the extensive use of NHP in techniques (particularly electrophysiological and anterograde tracing) which perturb neural activity, while defined circuits are engaged in specific cognitive tasks. There is also evidence for the involvement of the rodent cerebellum in cognitive flexibility and spatial navigation and some evidence in support of engagement in working memory and discrimination learning.[122-124] The availability of genetically modified lines and viral vectors, combined with optogenetic approaches, facilitate cell-type/population specific expression under tight temporal control.
3. *Behavioral studies*. Researchers have developed an array of behavioral paradigms that allow complex functions to be directly mapped to the activity of defined neuronal populations, identified to a high degree of functional, genetic and anatomical precision.[125] These paradigms have been validated by meeting the essential criteria of face validity (phenomenological similarity of the animal condition to the condition it is intended to model), construct validity (a theoretical rationale underlying the model consistent with current knowledge of the human condition) and predictive validity (manipulations known to influence the human condition, such as drug effects, have an equivalent effect on the model).[126-129] Coupled with technological advances already mentioned, behavioral studies provide powerful insight into brain networks. In this context, we and others have identified anxiety-like behavior prior to the earliest detectable accumulation of autoreactive T cells in EAE[90,91] in evaluation of depressive-like behavioral syndrome due to MS.

We have been unable to find reports of combined EAE cerebellar pathology/CNS circuitry; however experimental paradigms have been described which may provide such opportunities. One example is a study demonstrating the relationship between altered expression of Purkinje cells components and motor circuitry. As described above,[130] shifting expression patterns of mGlu1 and mGlu5 on Purkinje cells is a feature of both MS and EAE, associated with impaired motor control in EAE. In MS, these abnormalities are known to be related to altered conditioned eyeblink reflex, a test detecting cerebellar involvement in CNS neurodegenerative conditions.[131] This reflex generated by a small air puff to the cornea, co-incident with a stimulus such as tone, depends upon the cerebellum, reflecting the development of associative memory within the cerebellar motor circuitry. It would be of interest to investigate mGlu1 and mGlu5 conditional deletion mutants in the presence of EAE, to further map damaged/altered circuitry relating to motor control and gain insight into the condition eyeblink reflex and its clinical relevance. An additional model which may reveal important information regarding circuitry is the *CPT1AP479 L* conditional gene deletion mutant,[132] generated to investigate the potential protective effect of the carnitine palmitoyl transferase (CPT1A) gene in

the Inuit population (carrying a proline to leucine substitution in position 479 [*P479 L*]), of low MS incidence. CPT1A is a key lipid metabolism component. EAE induction in these mice was associated with significantly reduced disease severity under conditions of either high or low fat diets, whereas WT counterparts exhibited severe disease, exacerbated by a high fat diet. Therefore the mutation is associated with downregulation of lipid metabolism and resistance to EAE. A surprising observation was the protection from demyelination observed in the mutant, in the cerebellum and brainstem (ventral spinocerebellar tract, pyramidal and inferior olivary complexes). It would be of interest to further analyse cognitive performance in this mutant to explore the connection between diet, cognitive impairment and alterations in cerebro-cerebellar connectivity. Similarly, instances of combined optogenetics/EAE cerebellar pathology investigations are not available. However, the generation of a transgenic rat lines by lentiviral expression of either the light-activated cationic channel channelrhodopsin-2 (ChR2) or light-driven chloride pump halorhodopsin (eNpHR) under the control of the PC-specific L7 promoter have been described.[133] These models may provide novel opportunity to investigate Purkinje cell changes associated with EAE, as well as evaluation of candidate therapeutics.

Optimization of experimental autoimmune encephalomyelitis variants for pre-clinical and clinical evaluation of cerebellar dysfunction.

In view of the complexity of these investigations and costs involved, robust protocols will be required to ensure reproducibility of experimental conditions and sensitivity of parameters of interest. Below is a list of parameters which should be optimized prior to evaluation of cerebellar dysfunction, effects of candidate therapeutics, or for the identification of sensitive markers.

1. *Selection of appropriate EAE variant*. Because of the wide divergence in spatio-temporal lesion dvelopment and severity between variants, it is important to first ensure that the EAE variant of interest exhibits substantial cerebellar lesions with reproducible topography. Given that experimentation is generally performed using the spinal cord or whole brain, information regarding parameters of cerebellar disease is usually absent from the literature and will need to be generated. Additionally and perhaps counterintuitively, variants exhibiting chronic-relapsing or monophasic disease profiles are less useful than those exhibiting chronic-progressive profiles for evaluation of drug effects, because the spontaneous disease resolution allows only a very short time window of 5-7 days of experimentation in chronic-relapsing or monophasic, as opposed to 50 or even 80 days for chronic progressive disease.
2. *Cohort sizes*. Despite generally exhibiting synchronous clinical onset and peak of disease severity, wide between-subject variations are commonly observed with any given EAE variant. Therefore, n values of 4-6/group are appropriate only for large effects. A power analysis must be performed to determine the appropriate sample size. This may not be feasible if the variant of interest is a NHP, however.
3. *Pathological evaluations*. Detailed histopathological and immunochemical characterizations of should be performed, using techniques which are now well-established, to allow qualitative and quantitative determinations of pathological hallmarks.
4. *Biochemical profiling*. Immune cell, as well as inflammatory signature molecule profiling should be performed. These can easily be done at reasonable costs using commercially available kits.
5. *Evaluation of functional disturbances*. A wide range of validated tests for motor or cognitive/memory/emotional state is available. In rodents, they are more commonly used in rats rather than mice, because of the perceived higher intelligence and decision-making ability of rats. Testing should be performed with the collaboration of behavioral experts because of the complexity of data analysis, especially when dealing with NHP. The timing of the tests is also of critical importance[90,91] and should not rely on clinical scoring of ambulatory difficulties which are a reflection of spinal cord rather than cerebellar damage. They must be based on prior establishment of the spatio-temporal pattern of disease development specifically in the cerebellum.
6. *Candidate drug evaluation*. Based on the above parameters, determination of disease development on cerebellar function and candidate drug evaluation will generally rely on rodent EAE, with NHP as a second species. They will require accurate determination of cohort sizes. The timing of drug delivery and motor or behavioral testing will be determined based on pathological evidence of disease development/resolution and final evaluation will be supported by flow cytometric, biochemical and histopathological quantitative data.

Conclusions

Clinical and pathological evidence of early cerebellar damage in MS together with the recent appreciation of the role of this region in non-motor functions suggest that greater focus on the cerebellum will provide insight into processes underlying disease progression. This will be facilitated by combined advances in imaging technology and methods to investigate brain circuitry and cognitive processes. Additionally, improved use of animal models should be adopted, with EAE being viewed not as a disease recapitulating MS, but as an experimental paradigm allowing exploration of candidate mechanisms. Investigators should capitalize on the potential for genetic manipulation provided by rodent EAE, as well as the greater validity of NHP EAE for translational studies. This could potentially lead to the identification of sensitive markers

of disease progression and offer early points of intervention, as well as improve evaluation of candidate drugs aiming to protect against neurodegeneration.

Authors' contributions

DLM wrote the first draft of the article and generated the figures. DLM and JMO contributed equally to the final version.

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Appendix

Abbreviations

BBB	Blood brain barrier
CNS	Central nervous system
DCN	Deep cerebellar nuclei
GM	Grey matter
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NAWM	Normal appearing white matter
NHP	Non-human primates
Pm	Post-mortem
PPMS	Primary progressive multiple sclerosis
RRMS	Relapsing-remitting multiple sclerosis
WM	White matter