Teaching Course 13

Myelin regeneration and neuroprotection in multiple sclerosis: from cellular mechanisms to clinical trials

Chairs:  
C. Lubetzki (Paris, FR)  
B. Stankoff (Paris, FR)

35 Experimental approaches to investigate the mechanisms of remyelination in the CNS  
C. Lubetzki (Paris, FR)

36 Neuronal damage and perspectives of neuroprotection in MS  
D. Mahad (Edinburgh, GB)

37 Imaging tools for clinical trials of remyelination and neuroprotection  
B. Stankoff (Paris, FR)
Experimental approaches to investigate the mechanisms of remyelination in the central nervous system

Catherine Lubetzki
Hôpital de la Salpêtrière, Brain and spinal cord Institute, University Paris 6, Paris, France

To promote remyelination in multiple sclerosis, and therefore prevent degeneration of naked axons, several avenues are actively explored, focusing either on exogenous repair through graft of potentially remyelinating cells, or on endogenous repair, by favouring the existing, albeit often insufficient remyelination capacity of the adult central nervous system.

In this context, the use of experimental models is crucially needed to gain insight into the cellular and molecular axo-glial mechanisms corresponding to the different steps of the repair process. These steps consists of the activation of the oligodendrocyte progenitor cells, which are then recruited to the demyelinated area, before maturing into oligodendrocytes and finally wrapping oligodendroglial processes along the naked axons. Deciphering these mechanisms will result (and has already resulted) in identification of potential therapeutic targets. These models also permit to evaluate the influence of inflammatory cells on the different steps of the repair process.

In addition, these experimental models are also needed to evaluate the efficacy of the different repair strategies: different models have been developed, from those used for high through put screening to preclinical models in different species.

We will review some of these models, giving some examples of results that were obtained using these different tools, and highlighting new developments.

IN VITRO MODELS OF MYELINATION

1) Primary dissociated central nervous system myelinating co-cultures, widely used, are useful to gain insight into mechanisms of myelination. These are usually obtained from embryonic central nervous system, and contain all cell types of the central nervous system. As examples, these cultures allowed to demonstrate that even in vitro, oligodendrocytes myelinate solely axons, suggesting the existence of an axonal recognition signal (Lubetzki et al 1993). The same approach showed that electrical activity of neurons was necessary for the initiation of the myelination process (Demerens et al 1996). In these cultures, myelination is assessed by counting the number of myelinated internodes. This is time consuming, and not well adapted to screening myelinating activity. In this context,
luminometric assay of beta-galactosidase activity from myelinating co-cultures derived from MBP-<i>lacZ</i> lines (in which the lacZ transgene is under the control of the myelin promoter MBP, myelin basic protein), has been developed as an index of myelination (Stankoff et al 2002) and used, as a medium-through put screening to assess promyelinating activity. Using this method, it was shown that both CNTF related molecules and sigma receptors agonists positively influence myelination.

2) Dorsal root ganglia neurons and oligodendrocytes co-cultures. Although dorsal root ganglia neurons are not “central nervous system only” axons, as they also run into the peripheral nervous system, these cultures are useful as they allow to analyze separately the neuronal and oligodendroglial factors, through cultures in which neurons and oligodendrocytes are obtained from different sources and can be manipulated independently. Using this model, the Ffrench Constant group analyzed the influence of different mitogenic growth factors on myelination (Wang et al, 2007)

3) Mouse pluripotent stem-cell-derived oligodendrocyte progenitor cultures. The induced-pluripotent stem cells (iPS) technology allows to develop oligodendroglial cells from skin fibroblasts. These cells can then be transplanted in a demyelinated area to promote remyelination. In addition, this iPS technology has been developed to screen promyelinating activity. As an example, the Tesar group recently screened a library of bioactive small molecules. This resulted in the identification of seven drugs, which at nanomolar concentration selectively enhance the generation of mature oligodendrocytes from progenitor cells in vitro (Najm et al 2014). Among these, 2 drugs, miconazole and clobetasol, were further shown to impact myelination in cerebellar slice cultures

4) Engineered micropillars for high through put screening
Recently the Chan group (Mei et al, 2014) developed a binary indicant for myelination using micropillar arrays (BIMA) engineered with conical dimensions. Confocal imaging acquired from the base to the tip of the pillars allow for detection of concentric wrapping observed as 'rings' of myelin. The platform is formatted in 96-well plates, amenable to semi-automated random acquisition and automated detection and quantification. This methodology resulted in the identification of a cluster of antimuscarinic compounds that enhance oligodendrocyte differentiation and (re)myelination.

EX VIVO MODELS OF (RE)MYELINATION : the slice cultures
A disadvantage of the above described in vitro models is that the three dimensional structure of the tissue is absent. An alternative is the use of slice cultures, which are grown on semi-
porous membranes. Although myelination on slices can be obtained from different central nervous system areas, the cerebellar slices have been widely used for studying myelination (see review by Jarjour et al 2012). Direct visualization of myelinating cells can be achieved by immunohistochemistry, or by detection of the transgene (GFP) when slices are prepared from mice expressing a fluorescent reporter under the control of an oligodendrocyte promoter. Demyelination can be achieved using antibodies or myelinotoxic agents such as lysophosphatidylcholine. In 2012, Anna Williams and colleagues reported the evidence of remyelination after toxic-induced demyelination, with thinner myelin sheaths and shorter internodes. This model was used to identify the pro-remyelinating capacity of retinoic acid by the Franklin group (Huang et al 2011). In addition, they developed an interesting automated method of quantifying myelination by measuring the colocalization of MBP (myelin basic protein) immunofluorescence with that of the high molecular weight neurofilament subunit (NFH) (Zhang et al, 2011). This ex vivo model can be used to study the dynamic of myelination and remyelination by time-lapse imaging using fluorescent and 2-photon microscopy.

Alternatively, another ex vivo model of myelination well suited for dynamic imaging was developed by the group of Sue Barnett using pieces of intact spinal cord (Ioannidou et al 2015)

IN VIVO MODELS TO ASSESS MYELINATION AND REMYELINATION

These in vivo models have been developed in different animal species, using different ways to induce demyelination and/or oligodendrocyte loss. Aside from demyelinating models, inflammatory models are of interest, to better mimic the different components of multiple sclerosis pathogenesis.

1) Murine models
   - Toxic models

These models, in which demyelination is either induced by stereotactic delivery (lysophosphatidylcholine or ethidium bromide) or dietary ingestion of a demyelinating agent (cuprizone) are classical models of demyelination. As examples, these models have been used in pre-clinical studies to establish the pro-remyelinating efficacy of anti-Lingo monoclonal antibodies (Mi et al, 2009). Five weeks cuprizone exposure is mostly inducing a demyelination restricted to the corpus callosum, but recent developments have shown that adding the rapamycin agent was increasing the extent of the demyelinated area. As these models remyelinate robustly, pro-remyelinating agents accelerate rather than increase repair rate (Piaton et al, 2011). Models in which repair is less optimal are therefore crucial
prerequisite for clinical trials. One strategy is to assess repair in old animals, but alternatively
models of chronic demyelination might be more appropriate to study the progressive phase
of MS. The identification of abnormally thin myelin sheaths (increased g ratio) remains the
most reliable means of unequivocally identifying remyelination, relying mostly on analysis of
semi-thin and ultrathin tissue sections.

- **Transgenic models**
  To gain insight into the consequences of oligodendrocyte loss, the Merson group has
generated a transgenic mouse model of conditional oligodendrocyte ablation. In this model,
oligodendrocytes are rendered selectively sensitive to exogenously administered diphtheria
toxin (DT) by targeted expression of the diphtheria toxin receptor in oligodendrocytes.
Interestingly the results show that oligodendrocyte loss induces profound axonal pathology
that occurs before the removal of the myelin membrane, i.e., in the absence of
demyelination.

- **Inflammatory models**
  The above models are well suited for assessing the repair process. If the results are
promising, validation of a given compound in an inflammatory model, closer to multiple
sclerosis pathology, is necessary. Experimental allergic encephalomyelitis (EAE) can be
elicited in many different species, including rodents and primates, with different antigens
(Gold et al 2008). The EAE model obtained by immunizing rodents with myelin
oligodendrocyte glycoprotein (MOG) is the most studied. Recently, focal variants of the EAE
model that results in immune-mediated demyelination at a predictable time and location have
been proposed, and might be more suitable for assessing repair (Merkler et al., 2006).

2) Myelination in the zebrafish
  Zebrafish, which are transparent, can be used to study central nervous system
myelination at single-cell resolution in vivo. Using this method, Simons and Lyons deciphered
some mechanism of myelin formation, such as the axonal selection by oligodendrocyte
processes (Simons and Lyons, 2013). This method is of great interest, although there are
some clear differences between mammals and zebrafish central nervous system myelin
molecular composition. In addition laser-induced demyelination of transgenic zebrafish larvae
can be useful for screening for potential pro-myelination compounds (Buckley et al 2010).

3) Studying remyelination in the Xenopus
  Tadpole larvae are also transparent, and an attracting species for studying myelination as
myelin is very similar in tadpole and mammals on the one hand, and transgenesis is highly
efficient on the other hand. Recently the Zalc group generated a Xenopus laevis transgenic line pMBP-eGFP-NTR (nitroreductase) allowing, by addition of metronidazole in the swimming water of tadpoles, a conditional ablation of myelinating oligodendrocytes, due to conversion of the innocuous prodrug to a cytotoxin by NTR. After cessation of metronidazole treatment, remyelination proceeded spontaneously. This method, that can be considered as a medium through put screening tool, has enabled to confirm the promyelinating activity of most of the published pro-myelinating compounds (retinoic acid as an example) and is currently actively used to screen large libraries of molecules for promoting repair.

4) Myelination in the non human primate
As experiments in non-human primates are an essential step towards clinical trials, models of lysophosphatidylcholine-induced demyelination have been developed. As examples, demyelination can be targeted to the macaque dorsal spinal cord and optic nerve, leading to functional deficits. Remyelinating efficacy is studied using clinical, neurophysiological markers in correlation with post-mortem analysis. As an example, Lachapelle et al (2005) have demonstrated the differential remyelinating capacity of spinal cord and optic nerve in the macaque. EAE models are also developed in marmoset, but are hampered by an acute course of the disease, not well adapted for the study of potential pro-remyelinating agents. Studies are ongoing to reduce pathologic severity by modifying immunization protocols.

In conclusion: The recent development of translational researches focused on strategies to favour remyelination has considerably reactivated this research field, and highlighted the importance of complementary models to study and evaluate myelin repair. These different and complementary tools has resulted in identification of new mechanisms and therapeutic targets for these actively emerging strategies.
REFERENCES


Teaching course 13

Neuronal damage and perspectives of neuroprotection in MS

1. neuropathological findings in MS

The principal neuropathological features of MS include inflammation (adaptive and innate immunity), demyelination (white matter and cortical), incomplete remyelination and damage to neuronal compartments (axon, dendrites, synapses and neuronal cell body)\(^1\). Whilst inflammation and demyelination, and associated axon pathology, dominate the early stages of MS, the neuronal compartments in this chronic inflammatory demyelinating disorder become increasingly compromised with time\(^2\). Furthermore, neuronal damage becomes more apparent in the non-demyelinated white matter and grey matter. In a subset of cases with an early onset and a rapid rate of progression, inflammation within meninges appear to play a part in the cortical pathology as well as the diffuse white matter pathology in the spinal cord\(^3\). A triple prong approach, whereby myelin is restored to demyelinated axons, adaptive and innate immunity in the central nervous system is modulated and the neuronal compartments as well as the contents are therapeutically target, is likely to offer the best chance of protecting CNS tissue and preserving neurological function in progressive MS\(^4\).

2. Axo-glial metabolic coupling

In myelinated axons, the vast majority of the axolemma is enclosed by myelin and, not surprisingly, recent high profile studies have identified evidence of a metabolic connection between oligodendrocytes and axons\(^5, 6\). For example, lactate produced by glycolytic oligodendrocytes, because of lack of cytochrome c oxidase or complex IV of the mitochondrial respiratory chain, appears to be transported to the axon via monocarboxylate transporters which can then be used to generate pyruvate for the tricarboxylic acid cycle within mitochondria in axons. The loss of myelin will disrupt this axo-glial metabolic coupling and limit
the supply of metabolic substrates from oligodendrocytes to axons within plaques in MS. Furthermore, a recent study of glucose and monocarboxylate transporters in MS tissue identified a reduction in the axonal lactate transporter in chronic inactive MS lesions.

3. Mitochondrial changes within neuronal compartments in progressive MS

Recent studies from our laboratory and by other independent investigators have established mitochondrial respiratory chain deficiency (complex I, complex III, complex IV and complex V) within neurons in progressive MS cases. Nearly all neurons in motor cortex in progressive MS cases (non-demyelinated grey matter) show a complex I and complex III deficiency, due to a decrease in a number of nuclear DNA encoded mitochondrial transcripts (enzymes are deficient rather than completely destroyed). Furthermore, a subset of neurons in deeper cortical layers (layer V and layer VI) contained clonally expanded mitochondrial DNA deletions at high heteroplasmy level.

In demyelinated axons, functional mitochondria have to gather in abundance to meet the increased energy demand resulting from the altered sodium channel distribution. This axonal mitochondrial response (increased size, number and activity) appears to be compensatory following the loss of myelin and metabolic support from oligodendrocytes (axo-glial metabolic coupling currently appreciated as lactate transport), at least in the short term. If myelin is not restored to the axon, the axonal mitochondrial response may eventually lead to the accumulation of dysfunctional mitochondria in chronically demyelinated axons, a mechanism proposed using shiverer mice lacking syntaphilin, axon specific mitochondrial docking protein. Ongoing work in our laboratory indicate the gathering of mitochondria in acutely demyelinated axon to be a consistent feature in experimental systems, independent of how the axons are demyelinated. However, the increased mitochondrial presence is not always mirrored by the activity of mitochondrial respiratory chain, in particular complex IV. For example, lysolecithin and ethidium bromide induced experimental demyelination leads to an increase in complex IV activity reflecting
intact function of the gathered mitochondria in the axon upon demyelination. In contrast, the majority of mitochondria within axons located within lesions in experimental autoimmune encephalomyelitis (EAE) lack complex IV activity, presumable because of acute damage from reactive oxygen species and inhibition by nitric oxide. The acute mitochondrial injury in EAE is evident in in-vivo imaging studies, carried out by Kerchensteiner’s group, and is associated with spontaneously reversible changes in axon morphology, irrespective of demyelination, termed focal axonal degeneration\(^{16}\). Furthermore, the same group described widespread axonal transport deficits in myelinated axons in EAE, which preceded damage to the axonal ultrastructure\(^{17}\). Factors that are extrinsic and intrinsic to neurons appear to play a role in the axonal energy failure in progressive MS\(^2\).

In progressive MS, the axonal mitochondrial response is no longer evident within the degenerating demyelinated axons in active and inactive lesions\(^{13}\). The axonal energy failure in progressive MS is likely to be, at least partly, because of the inability of the neuronal cell body to replenish the axon with healthy mitochondria, adversely affecting the demyelinated axons more than myelinated fibres (energy failure in neurons is due to a combination of inability to produce healthy mitochondria in the cell body and the increased demand for functional mitochondria by the demyelinated axon)\(^2\).

4. Limitations of established disease models

The existing disease models of MS do not adequately captured the neuronal cell body mitochondrial abnormalities, evident in the grey matter\(^8, 18\). As a result, the consequences of the neuronal mitochondrial respiratory chain defects for progressive MS, unlike for classic neurodegenerative disorders, are not well understood. Furthermore, the causes of mitochondrial abnormalities in progressive MS are not completely understood. There is a pressing need to model the mitochondrial deficits seen in MS, cortical pathology and chronic demyelination in vivo to understand the mechanisms of tissue damage in progressive MS and identify potential therapeutic targets.
6. therapeutic targets for progressive MS

It is clear that a multi-prong therapeutic approach is needed in progressive MS to modulate the immune response, encourage restoration of myelin to axons and protect neurons\(^4\). Potential therapeutic targets and agents to protect axons and neurons in progressive MS will be discussed at this workshop.
References


11. Fischer MT, Sharma R, Lim JL, Haider L, Frischer JM, Drexhage J, et al. NADPH oxidase expression in active multiple sclerosis lesions in relation to...


Advanced imaging tools for clinical trials of remyelination and neuroprotection

Bruno Stankoff a,b

a, ICM, Institut du Cerveau et de la Moelle épinière, Sorbonne Universités, UPMC Univ Paris 06, UMR S 1127, and CNRS UMR 7225, Hopital Pitié Salpêtrière;
b, APHP Hôpital Saint-Antoine, Paris, France

I. Introduction

During the past decades, multiple sclerosis (MS) has been an outstanding example illustrating the considerable contribution provided by imaging techniques, such as magnetic resonance imaging (MRI), to the improvement of clinical diagnosis and the design of clinical trials. Nowadays, the presence of MS white matter lesions on T1- and T2-weighted sequences, together with the pattern of contrast enhancement following gadolinium injection are key criteria to establish the diagnosis of MS 1. Furthermore, the dynamic evolution of lesions on sequential MRI scans is a strong predictor of the efficacy of disease modifying therapies on relapse rate 2. However, current immuno-active therapies still fail to prevent long-term disability progression, and imaging metrics derived from classical structural MRI are not yet fully predictive of disease progression. As a result, a growing interest has developed on investigating the neurodegenerative component of the disease, which could be related to key physiopathological mechanisms such as a failure of myelin repair, grey matter damage, and compartmentalized inflammation. Conventional MRI sequences, while very sensitive for the detection of lesional areas, are not specific for the underlying tissue damage, as they may reflect equally inflammation, oedema, myelin pathology and axonal degeneration. This has led to the development of many advanced imaging tools with an optimized specificity for the complementary facets of tissue damage in MS. In this review, we will describe advanced imaging techniques that have been proposed to investigate the myelin compartment and the grey matter damage that could be applied to therapeutical trials.

II. Imaging tissue damage 1: myelin imaging

Several advanced MRI sequences have been proposed to evaluate more specifically the myelin compartment within the CNS, such as magnetisation transfer imaging (MTI), T2 relaxometry, and diffusion tensor imaging (DTI) 3.

1. Magnetisation transfer imaging (MTI) is based on the interaction and exchange between the unbound protons in free water and the protons bound to macromolecules. Changes in the magnetization transfer ratio (MTR, which reflects the exchange rate of magnetisation transfer between the two proton pools) of cerebral white matter are thought to be highly influenced by changes in myelin content because of the overwhelming contribution of myelin to the macromolecules involved in the MT phenomenon. Therefore, a reduced MTR is likely to reflect loss of myelin whereas an MTR increase may indicate remyelination 4,5. MR-histopathology studies performed on post-mortem brains from MS patients have further supported an association between MTR measurements and myelin content 6. The MTR of remyelinated lesions differed from normal appearing white matter (NAWM) and demyelinated lesions, with a significant correlation between MTR and myelin content in lesions and in NAWM 6. In optic neuritis, MTR was shown to correlate with neurophysiological function scores, such as visual evoked potential latency and optical coherence tomography, a measure of retinal neuroaxonal loss 7. Several ways to quantify the MTR signal have
been investigated. The most commonly used relies on the measurement of the mean MTR within regions of interest (acute active lesions, T2 lesions). In longitudinal studies, follow-up MRI examinations have to be coregistered with the reference one, and the evolution of the mean MTR can be determined in each ROI. Using such a strategy, it has been shown that MTR decreases slightly before lesion appearance on T2-Weighted sequences, and then drops dramatically at the time of gadolinium enhancement. A partial recovery of MTR values in active lesions, suggestive of remyelination, has been observed from 1 month up to 6 month, a time frame compatible with what is expected from experimental studies. However, following optic neuritis, a slower decrease of MTR (nadir at 240 days after the clinical episode) has been observed, with only partial recovery after one year: a slow clearance of myelin debris in the optic nerve has been suggested to explain this finding, but remains speculative. As heterogeneity in the level and time course of demyelination and remyelination could occur between lesions and within each lesion, a voxel-based quantification method has been developed. A procedure for the application of MTI to clinical trials together with sample size estimation was recently proposed. Recently, MTI has further been shown to be sensitive to cortical pathology and a methodology to assess cortical demyelination at the individual level has been proposed. Overall, while being probably the most available MRI technique to approach the assessment of myelin content, to date the specificity of MTI for myelin is still suboptimal, as water content, inflammation and axonal damage still represent relevant contributors to the signal modifications.

2. Myelin Water Fraction imaging. Besides MT imaging, the multicomponent T2 analysis of spin echo data, by focusing on the short T2 peak between 10 and 50 ms, has been proposed to extract the myelin water fraction (MWF), which likely corresponds to water trapped within the myelin bilayer. The T2 decay in the brain presents a few peaks, which have been assigned to compartmentalized spin populations: the short T2 peak (around 20 ms, between 10 ms and 50 ms) represents the water trapped within myelin layers, the peak around 70-90 ms the intra and extra-cellular water, and the third peak above 2 sec reflects CSF water. Correlations between MWF and MTR measures within MS lesions and between MWF and luxol fast blue staining on histopathologic slices have been reported. As the reproducibility of MWF measure has been questioned using a ROI analysis strategy, a voxel-based analysis has been recommended. Few longitudinal data on lesional MWF are available, indicating that some changes compatible with remyelination could be identified in a 6 months time frame, but with a greater variability than using MT imaging. An optimization of these sequences, aimed to result in shorter acquisition times, increased sensitivity and better reproducibility, is required, and several adaptations of the concept have been developed in over recent years providing promising results in MS.

3. Diffusion weighted imaging (DWI), a measure of water molecules diffusion, has provided details on tissue microstructure and allowed to perform fiber tracking. The directional diffusivity derived from DTI measurements describes microscopic water movements parallel to (axial diffusivity) and perpendicular to (radial diffusivity) axonal tracts. In experimental models of white matter injury, the water radial diffusivity has been shown to reflect mainly myelin content, whereas axonal pathology was the main contributing component to the changes in axial diffusivity. Measures of radial diffusivity could predict further demyelination in ex-vivo MS spinal cords and has been able to discriminate the functional outcome following optic neuritis in humans. The longitudinal monitoring of MS lesions has shown reduction in radial diffusivity over time that might be compatible.
with remyelination\textsuperscript{42}. However, to date the measure of RD is not yet pathologically specific as it can be significantly influenced by axonal pathology and inflammation\textsuperscript{40}.

4. Molecular imaging of myelin by positron emission tomography (PET)

Despite the major advances in MRI techniques, there is no MRI technique specific enough for the assessment of myelin \textit{in vivo}. Therefore, it would be useful to develop a molecular imaging technique specific for myelin that would quantify CNS remyelination and further validate the MRI-based metrics. This could be achieved using positron emission tomography (PET). Of great interest was the identification of a newly synthesized fluorescent stilbene Congo red derivative, 1,4-bis(p-aminostyryl)-2-methoxy benzene, also named BMB, that selectively binds to myelin \textit{ex vivo} and \textit{in vivo}\textsuperscript{43}. This compound has allowed the detection of demyelinating lesions in a rodent experimental autoimmune encephalitis model of demyelination. On MS brain samples, BMB staining can differentiate remyelination in shadow plaques from either demyelinated lesions or normal-appearing white matter, suggesting that this biomarker could be used to quantify myelin loss and repair. Reinforcing the hypothesis that the common affinity of BMB towards amyloid plaques and myelin could be explained by a similar protein conformation in both structures\textsuperscript{43,44}, a specific interaction of BMB with the myelin basic protein has been recently described\textsuperscript{45}. Finally BMB was shown to cross the blood brain barrier and, when radiolabeled with carbon-11, to allow CNS myelin imaging by PET in non human primates\textsuperscript{43}. Interestingly, a similar affinity for CNS myelin was reported for several other stilbene Congo red derivatives, allowing to follow-up demyelination and remyelination in dysmyelinating models as well as in the cuprizone-induced experimental model, either by using the fluorescent properties of the compounds or by using molecular imaging \textit{in vivo}\textsuperscript{46-48}. On the basis of a common target expressed in amyloid plaques and myelin, we have found that other amyloid markers, related to thioflavinT, could also stain myelin and be used as PET radiotracer for myelin\textsuperscript{49}. Using the [\textsuperscript{11}C]-2-(4'-methylanilinophenyl)-6-hydroxybenzothiazole ([\textsuperscript{11}C]-PIB), a proof of concept PET imaging study in MS patients has shown that this tracer allowed to visualize demyelinated MS lesions, with a less pronounced reduction of the uptake in gadolinium enhanced lesions than in non active lesions. This imaging probe has recently been investigated in a longitudinal study and could allow to classify patients depending on their remyelinating capacity (Bodini et al, unpublished). Other families of compounds, either related to coumarin\textsuperscript{50} or to the sphingosine-1-phosphate receptors (especially S1P5 that is oligodendroglial specific)\textsuperscript{51}, are now being developed for myelin imaging with PET. However, for all these compounds, further data are required to detail the specificity toward the myelin target, the binding saturability, and the signal-to-noise ratio on PET imaging.

III. Imaging Tissue damage 2: grey matter pathology and neurodegeneration

The most validated imaging metrics that could be applied to therapeutical trials aimed at promoting neuroprotection rely on dynamic volumetric measures of the CNS, in particular grey matter volume assessment. The reproducibility of such volumetric quantification is well characterized and acceptable for medium size sample studies, and the number of subjects to be included in trials has been defined\textsuperscript{52}. The prognosis value of atrophy rate has also been shown. However CNS volume loss measures have no specificity regarding the underlying tissue damage mechanisms, and a wide range of advanced imaging techniques has been developed to provide more specific tools for pathophysiologic mechanisms of neurodegeneration.
1. Cortical lesions detection.

Several cortical lesion subtypes have been identified during MS course, which could drastically contribute to neurological disability. Therefore newer MR sequences with improved contrast for cortical lesions such as DIR, MPRAGE, or PSIR have been implemented to enhance cortical lesions detection. Using these metrics in a clinical setting was shown to improved diagnosis criteria and to better explain cognitive deficits. However, to date, these sequences only detect about one-third of whole cortical lesions when applied on clinical scans (either 1.5 or 3T), and they usually miss the visualisation of subpial demyelination. This insufficient sensitivity might not yet allow us to perform accurate regional correlations between cortical lesion load and other MRI parameters or clinical symptoms.

2. Quantitative mapping of $T_2^*$ in the cortex

One major asset provided by ultra-high resolution (7 T) MRI is the high spatial resolution needed to quantify cortical degeneration. Due to the thin (~3.5 mm) and folded structure of the cortex, developing reliable measures of cortical tissue contrast still remains a technical challenge.

Recent ex-vivo and in-vivo imaging studies have explored contrasts underlying myelin and iron concentration in the cortex of healthy subjects and patients with MS. Deistung et al. were able, using susceptibility and $R_2^*$ (=1/$T_2^*$) mapping, to image ex-vivo laminar substructure of the cortex such as the Stria of Gennari in the primary visual cortex. Unlike $T_2^*$-weighted signal, $T_2^*$ relaxation time is a quantitative measure computed from several $T_2^*$-weighted images acquired with different echo times, thus independent from imaging parameters such as coil sensitivity or scaling factor. In healthy brain, $T_2^*$ relaxation time inversely correlates with myelin and iron content. Both in white matter and cortical MS lesions, histopathological-MR correlations demonstrated that increased $T_2^*$ relaxation time corresponded to areas of demyelination and iron loss, while iron accumulation at the border of the lesions induced shorter $T_2^*$.

Imaging subpial demyelination, a major pathological substrate of disease progression in MS, is one of the most important advance offered by quantitative $T_2^*$ imaging at 7 T. $T_2^*$-weighted and multi-echo $T_2^*$ imaging has allowed to increase the sensitivity to detect cortical lesions, including the subpial type, with an accuracy of 50% to 90% compared to histological examination.

Using tools from FreeSurfer, an imaging software designed to identify cortical surfaces, Cohen-Adad et al. detected $T_2^*$ signal abnormalities in the cortex of patients with MS. More recently, Mainero et al. were able to map $T_2^*$ relaxation time in the cortex of subjects with MS, at different depths through the width of the cortical ribbon, from the pial surface to the WM. The authors found a gradient of increased $T_2^*$ relaxation time (underlying myelin and/or iron loss) across all disease stages, correlating with neurological disability, with the most dramatic changes observed in the outer cortical layers, close to the pial surface as described in neuropathological studies.

In order to depict the independent contribution of myelin and iron content to cortical degeneration, other MRI contrasts can prove useful to explore subpial pathology. These include magnetization transfer imaging and $T_1$ mapping. In future, combining different contrasts could help to define more specifically the mechanisms at stake in disease progression.

3. Sodium imaging
In the past decade, experimental studies have highlighted sodium metabolism dysfunction as a mechanism of MS pathogenesis. Increased intracellular sodium accumulation, likely secondary to mitochondrial impairment, leads to neuroaxonal dysfunction and death. Imaging sodium concentration in the brain can therefore prove useful as a marker of neuronal dysfunction and loss in vivo in MS.

Clinically feasible Na MRI has been developed over recent years, this technique being previously limited by low signal-to-noise ratio and long scan time. Total sodium concentration has been quantified in several brain tissue compartments in patients with MS, and was increased in white matter lesions, normal appearing white matter, cortical and deep grey matter, throughout all disease stages. Moreover, total sodium concentration was higher in secondary progressive patients compared to relapsing-remitting and primary-progressive MS, and correlated with clinical disability. Future perspective include the development of novel techniques able to selectively quantify intra- and extracellular sodium concentration independently of each other, and to relate this biomarker to other functional imaging modalities to better understand the impact of sodium accumulation to neuronal dysfunction in-vivo.

4. Proton MR spectroscopy (MRS) allows the identification and quantification of brain and spinal cord metabolites, with the N-Acetyl-Aspartate (NAA) being specific for the axonal compartment. A decrease in NAA in patients compared with healthy controls has been reported in all phases of the disease, as soon as the radiologically isolated syndrome, indicating that neuronal dysfunction and/or axonal loss occur as soon as the earliest stage of MS. NAA changes have been correlated with neurological disability and cognitive deficits, attesting that neuro-axonal pathology is a crucial mechanism underlying disability in MS. MRS also allows the identification of other relevant metabolites such as glutamate and GABA, that could provide insight into neurodegenerative processes. In particular, glutamate levels measured by MRS at 3T was shown to be increased in NAWM and acute lesions, with no significant elevation in chronic lesions. However, technical limitations for the quantification of MRS peaks as well as a suboptimal spatial resolution still limit a large clinical use of this method. A promising emerging sequence relies on the combination of MRS and DWI (diffusion weighted spectroscopy), which allows to quantify the diffusivity of the main brain metabolites identified by proton spectroscopy. A pilot DWS study shows that the diffusivity of N-Acetyl-Aspartate was reduced in the corpus callosum of MS patients, a finding that could reflect the early and potentially reversible functional axonal damage associated with MS.

5. PET with the neuronal specific radiotracer [11C]-flumazenil could also contribute to assess the neuronal component of grey matter pathology in MS. Flumazenil binds to the benzodiazepine site contained within the GABA-A receptor, which is widely expressed by neurons in cortical and deep grey matter regions. A key point is the availability of a robust absolute quantification methodology based on the co-injection of labeled and unlabelled flumazenil, the partial saturation protocol, which provides an absolute quantification of GABA-A receptor concentration at the voxel level. Using a high resolution research camera it has been possible to quantify neuronal damage, which occurs as soon as the relapsing-remitting stage, and to localize the cortical regions where neuronal damage predominates. Interestingly, this recent pilot study suggested that neuronal damage was associated with clinical cognitive impairment, but also that it was not the only pathologic event contributing to brain grey matter atrophy.
IV Conclusions

Structural MRI is nowadays part of the routine procedures used for MS diagnosis and follow up in a clinical setting. Besides clinical detection of MS lesions, there are now a wide range of advanced tools that could contribute to understand MS physiopathology and to design future trials, aimed at enhancing myelin repair and reducing neurodegeneration and neuroinflammation. The implementation of specific imaging metrics and their application in MS, together with the development of appropriate post-processing methodologies, therefore represent an outstanding field of research that will contribute to improved knowledge and clinical care in the MS field\textsuperscript{81}. 
Bibliography


