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Review Article

Imaging the execution phase of neuroinflammatory disease models

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ABSTRACT

In vivo imaging of the rodent spinal cord has advanced our understanding of how resident cells of the central nervous system (CNS) respond to neuroinflammation. By combining two-photon imaging and experimental autoimmune encephalomyelitis (EAE), the most widely used rodent model of multiple sclerosis (MS), it has been possible, for example, to study how axons degenerate when confronted with inflammatory cells, how oligodendrocytes get damaged in inflammatory lesions, and how immune cells themselves adapt their phenotype and functionality to the changing lesion environment. Similar approaches are now increasingly used to study other forms of neuroinflammation, such as antibody/complement-mediated neuromyelitis optica spectrum disease (NMOSD). To tackle the most pressing open questions in the field, new biosensors and indicator mice that report the metabolic state and interaction of cells in neuroinflammatory lesions are being developed. Moreover, the field is moving towards new anatomical sites of inflammation, such as the cortical gray matter, but also towards longer observation intervals to reveal the chronic perturbations and adaptations that characterize advanced stages of MS.

1. Introduction

Multiple sclerosis (MS) is the most common non-infectious neuroinflammatory disease of the central nervous system (CNS). The disease is a major cause of disability affecting more 2 million people worldwide and resulting in immense personal and socio-economic disease burden (Reich et al., 2018). MS presents clinically as a multifaceted disease, characterized by a wide variety of neurological symptoms and a highly variable clinical course. While historically considered a demyelinating disease of white matter, caused by local immune infiltrating and resulting in axonal injury, it has become apparent that MS cannot be considered as just the sum of the white matter lesions. Rather, it is a disease of systemic aetiology with genetic and environmental risk factors and global effects throughout the CNS, including the gray matter (Baecher-Allan et al., 2018; Calabrese et al., 2015; Tremlett et al., 2017). The peripheral immune mechanisms and infiltration pathways are increasingly understood (Dendrou et al., 2015; Hemmer et al., 2015). Indeed, all of the approved MS therapies that have emerged over the past 25 years target these immunological aspects of the disease and are most effective in its early stages (Dendrou and Fugger, 2017). For the later stages of MS, which are often characterized by progressive disability, substantial therapeutic challenges remain (Salvetti et al., 2015). Indeed, in contrast to the immune interactions that characterize the initiation phase of the disease, the mechanisms of resulting tissue damage remain largely enigmatic. Still, over the recent decade, there has been substantial progress towards elucidating the cellular interactions and dynamics inside the inflamed CNS parenchyma that ultimately result in neuroinflammatory tissue damage. *In vivo* imaging techniques in rodent MS models have made an important contribution to this field. In this review, we compile the insights that many labs, including our own, have been able to glean from such studies, especially by using spinal cord *in vivo* imaging (Misgeld et al., 2007). This approach has revealed the behavior and interaction of a variety of cells (such as neurons, oligodendrocytes, immune cells) at

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different levels (whole cell behavior, subcellular dynamics, molecular changes) in order to resolve the events that are crucial in the initiation of a neuroinflammatory lesion and the subsequent induction of CNS tissue damage. In addition to MS, other diseases, although clinically similar, but pathomechanistically distinct neuroinflammatory entities, such as neuromyelitis optica spectrum disorder (NMOSD), are increasingly moving into the focus of *in vivo* animal studies. As the most prominent NMOSD subgroups have a relatively well-defined autoantigen and are in their initiation phase determined by the humoral branch of the immune system, comparing the initiation and execution phases of MS and NMOSD models can provide interesting insights into shared and distinct aspects of these disorders. Here, we will focus our attention on the study of execution mechanisms, while Borjini et al. in this issue will discuss the initiation mechanisms of CNS inflammation.

2. Animal models of CNS inflammation

Before considering *in vivo* imaging studies of spinal cord damage and their results and relevance to MS, it is essential to understand the model systems used for different studies and scrutinize their validity and utility. Experimental autoimmune encephalomyelitis (EAE) is an established and widely used model of MS.

EAE models, mostly induced in rodents and small primates, provide a set of tools to study CNS inflammation. Indeed, in most cases the inflammation in EAE is induced by injecting a purified myelin antigen or by transferring T cells reactive for these antigens (Ellwardt and Zipp, 2014). However, since its first introduction 85 years ago (Rivers et al., 1933), a debate has been ongoing regarding the usefulness of EAE in understanding MS and in predicting the success of treatment strategies (Sriram and Steiner, 2005). Still, in contrast to many models of neurodegeneration, EAE has already been instrumental in devising effective treatments for MS, such as glatirameracetat and natalizumab (Steinman and Zamvil, 2006). Thus, while no researcher will claim that EAE is a perfect mimic of MS, it is the central model in trying to visualize and understand the cellular dynamics of neuroinflammatory lesions.

Several considerations guide the use of EAE for *in vivo* modelling of MS. First, because of its somewhat artificial modes of initiation, unpredictable disease course and strong dependence on environmental factors, such as animal stress levels or microbiome composition (Berer et al., 2011; Gerrard et al., 2017), EAE might appear as a cumbersome animal model. However, these features represent the reality of neuroinflammation. Indeed, EAE – like MS – is a systemic disease that we cannot reconstitute easily in reductionist systems or model organisms. Hence, for now EAE models remain a powerful tool to study the complex immunology and neurobiology of the underlying disease mechanisms. This being said, the importance of guiding EAE studies by information obtained from human immunology, genetics and neuropathology is obvious.

Second, there is no singular and supreme form of EAE. Rather, EAE represents a group of autoimmune demyelinating diseases that can be induced in a variety of experimental animals (*e.g.* mice, rats, marmosets) and are heavily influenced by genetic background, resulting in a variety of disease courses, including monophasic, chronic relapsing-remitting or progressive (Krishnamoorthy and Wekerle, 2009). While none of these models perfectly reproduces the temporal and spatial characteristics of MS, the variety of EAE models is also a strength. For instance, we can tailor the model to represent different aspects of the disease and study them in relative isolation. Moreover, we can validate experimental findings across different variants and species to find mechanisms that are more likely to be conserved in the human disease.

Third, three fundamentally different modes of EAE induction exist and prove more or less suitable for specific types of studies. “Active” (aEAE) and passively transferred forms of EAE (pEAE) differ in their mode of induction. aEAE involves active immunization using neural antigens, typically derived from myelin, but also of astroglial and neuronal origin, mostly delivered in complete Freund adjuvants (CFA). This immunization induces a strong autoimmune reaction that - depending on species and background - results in mono- or multiphasic limb paresis. These models are technically easy to induce, including in non-human primates (Krishnamoorthy and Wekerle, 2009), and are the most commonly used models to study axon degeneration in the murine spinal cord (Nikić et al., 2011; Sorbara et al., 2014). pEAE involves *in vitro* activation and transfer of CNS-specific antigen-specific T cells into the model organism to induce a relapse. Most studies addressing mechanisms of immune cell infiltration across the blood-brain barrier have used this mode of EAE induction (Bartholomäus et al., 2009; Schläger et al., 2016). In addition, a third group of EAE has been established, which can be considered “spontaneous” (sEAE) and does not involve immunization or cell transfer, but genetic manipulations such as overexpression of TNF-α or other cytokines in CNS cells or the introduction of CNS-specific receptors and autoantibodies into the immune repertoire (Krishnamoorthy et al., 2007). Such sEAE models provide the opportunity to study immune determinants of disease onset and course, such as regulatory T cell interactions, but will likely also become important models to study tissue injury.

While in the literature MS models are represented in abundance, for NMOSD the choice of models is much more limited. Even though first transfer models of NMOSD have been described (Li and Yan, 2015; Wu et al., 2018), their mode of induction is rather complicated and their clinical picture remains comparatively far from the human disease. Instead, most experimental studies use direct application of a putatively pathogenic mix of human-derived autoantibodies and complement to induce NMO-like lesions (Bradl et al., 2018). At least at the histopathological level, this approach results in lesions that bear some similarity with the corresponding human conditions. Recently, we have adapted this approach to model subpial NMOSD pathology in the murine spinal cord within reach of intravital imaging (Herwerth et al., 2016).

3. Insights gleaned from *in vivo* imaging

Spinal cord *in vivo* imaging has provided a number of new insights into the pathophysiology of neuroinflammation revealing cellular interactions, organelle dynamics or even molecular effector cascades. Here, we will focus on putative execution mechanisms of immune-mediated nervous system damage that are activated in spinal neuroinflammatory lesions. The processes that lead to the initiation of such neuroinflammatory lesions, including the activation of immune cells and their trans-migration across the blood-brain barrier, are discussed in detail in another article of this special issue (Borjini et al.).

4. Phagocyte actions in neuroinflammatory lesions

After adaptive immune cells initiate the formation of lesions in MS and its models, innate immune cells such as CNS phagocytes play a central role during subsequent lesion expansion and resolution. These CNS phagocytes are comprised of resident microglia, perivascular macrophages, as well as choroid plexus and meningeal macrophages, complemented by infiltrating monocyte-derived phagocytes (Henderson et al., 2009; Li and Barres, 2018).

While novel approaches such as single cell RNAseq and CyTOF begin to provide a highly resolved view of the molecular complexity of CNS phagocytes in the healthy and inflamed CNS (Ajami et al., 2018; Sousa et al., 2018), we still lack a clear understanding of their *in vivo* actions and interactions, providing the rationale for intravital imaging. To image interactions of a specific cell type in complex tissue, first, there has to be a suitable labelling strategy. A breakthrough in observing microglia was achieved with the development of the Cx1cr3$^{GFP/+}$ mouse line, with a GFP knock-in replacing one allele of the fractalkine receptor. This genetic manipulation results in a strong labelling of microglial cells up to their finest processes (Jung et al., 2000). Capturing

the activity of microglia in the living brain using two-photon microscopy has demonstrated the dynamic capacity of these cells to scan surrounding tissue in the resting state and initiate a targeted response to stimuli (Davalos et al., 2005; Nimmerjahn et al., 2005). The same labelling strategy was also used to visualize the microglial response during the formation of neuroinflammatory lesions: Davalos and colleagues could show microglial clustering around vessels in the spinal cord, which was triggered by the plasma protein fibrinogen before clinical disease commenced. By interfering with the fibrinogen recognition by the integrin receptor on microglia, they could prevent this clustering. These microglial "pre-lesions" seem to precede local axonal damage and production of reactive oxygen species release as the disease progresses (Davalos et al., 2012). However, these damages were shown to be drastically reduced by the administration of fibrin-targeting immunotherapy in animal models of MS and Alzheimer's disease (Ryu et al., 2018) opening therapeutic perspectives around fibrinogen reactivity.

While microglia as well as their bloodborne relatives play a key role in lesion formation, the same cell types can also contribute to lesion repair processes (Ajami et al., 2011; Kroner et al., 2014; Miron et al., 2013; Weber et al., 2007). A concept that can explain these divergent actions of the same cell type stipulates that phagocytes can acquire distinct polarization (Hu et al., 2015). A transgenic labelling technique with two fluorescent proteins mirroring the intrinsic expression of pro- and anti-inflammatory signature enzymes recently enabled capturing this polarization in pro- and anti-inflammatory phagocytes in real time in the spinal cord (Locatelli et al., 2018). In these reporter mice, tdTomato is expressed under the iNOS promoter therefore representing the proinflammatory phenotype, whereas YFP is expressed under the Arginase promoter, a marker enzyme for tissue repair. *In vivo* imaging of the respective fluorescence protein expression could then be used to track the polarization of individual phagocytes. Extracting both spatial and temporal information, this study revealed that the local microenvironment seems to be a major factor in shaping the phenotypic differentiation: while phagocytes adopt a proinflammatory phenotype after entry in the spinal cord, these cells locally switch from a pro- to an anti-inflammatory phenotype as lesions start to resolve.

5. Glial targets of neuroinflammation

Oligodendrocyte damage and repair is a central feature of MS and its animal models (Bjelobaba et al., 2018; Wolswijk, 2000). One important question is where the damage is initiated within the oligodendrocyte, *i.e.* whether oligodendrocytes are attacked peripherally at the internodes that they sustain, or rather from their soma. A recent *in vivo* imaging report from our lab indicates that the destruction of this morphologically complex cell proceeds from the internodes, with only the closest processes to the cell body remaining just few days after the onset of EAE in spinal cord (Romanelli et al., 2016). In this constellation, the loss of myelin preceded the demise of the oligodendrocyte cell body. Even at the peak of EAE, Romanelli and colleagues observed that while myelin was almost undetectable, half of the oligodendrocyte cell bodies remained, leading to an 'amputated' oligodendrocyte morphology within inflamed lesions of the spinal cord. These findings corroborated earlier observations of oligodendrocytes showing a non-ramified morphology in the glial scar area of chronic demyelinated human MS lesions (Wolswijk, 2000). This 'centripedal' spread of oligodendrocyte damage further suggests that the initial steps of the destructive process target the myelin sheath. *In vivo* imaging of myelin sheaths using lipophilic fluorescent dyes (Romanelli et al., 2013) then revealed the formation of 'myelinosomes', *i.e.* myelin outfoldings, as an early event of immune-mediated oligodendrocyte damage. Further experiments showed that anti-myelin antibodies that bind complement and thus opsonize the internode surface for phagocytes are a key driver of myelinosome formation. In line with these findings, IgG1 monoclonal recombinant antibodies (rAbs) from clonally-expanded plasmablasts, recovered from the cerebral spinal fluid of MS patients, were also shown to drive complement-dependent demyelination and could be contributors to type II MS lesion pathology (Liu et al., 2017), suggesting a prominent role of the complement in demyelination during EAE and MS.

While oligodendrocytes and myelin are the main glial targets of immune-mediated damage in classical MS models, astrocytes are prominently affected in neuromyelitis optica spectrum disease (NMOSD) and its animal models (Li and Yan, 2015). For instance, bath application of human aquaporin-4 (AQP4) antibodies, characteristic for NMOSD, together with a suitable complement source cause immediate dose- and titer-dependent astrocyte toxicity and depletion in the mouse spinal cord (Herwerth et al., 2016). This depletion of astrocytes has profound effects on the other CNS resident cells, including oligodendrocytes and axons, likely due to the loss of the crucial trophic and barrier functions provided by these cells (Sofroniew, 2015). Using a co-culture system, it was further suggested that the damage to oligodendrocytes, which do not express AQP4, and the following demyelination inherent to NMOSD (Parratt and Prineas, 2010) were a bystander effect mediated by complement membrane attack complex (MAC) released by the dying astrocytes (Marignier et al., 2010; Tradtrantip et al., 2017). Thus, the complement pathway activation has emerged as a critical player both for myelinosome formation and myelin removal in MS models, as well as for the astrocytic toxicity and its downstream effects in NMOSD models (Herwerth et al., 2016; Romanelli et al., 2016). While acute intravital imaging provides the means to study the glial cell damage at the peak of inflammation, a repetitive imaging approach would need to be applied to monitor the processes and identify the players involved in the resolution and repair phases. This approach was recently used to investigate myelin dynamics in the mouse cortex using repetitive imaging over several months (Hill et al., 2018; Hughes et al., 2018).

6. Cellular, subcellular and molecular mechanisms of immune-mediated axon damage

While damage of axons is a well-characterized histopathological feature of MS lesions, the causative sequence underlying this process has not been identified (Trapp and Nave, 2008; Trapp et al., 1998). The advent of two-photon microscopy (Helmchen and Denk, 2005) and the availability of transgenic *Thy1*:XFP animals with selective axonal labelling (Feng et al., 2000), however, provided the technical means to reveal the initiation events of immune-mediated axon damage in MS models *in vivo* (Misgeld and Kerschensteiner, 2006). For example, using a MOG-induced active EAE model in *Thy1*:XFP transgenic mice, we were able to characterize the sequence of events resulting in axon damage within spinal cord lesions, a process we termed focal axonal degeneration (FAD) (Nikić et al., 2011). Two important insights emerged by observing the underlying morphological changes over time: first, the local swelling of axons represents a comparably stable intermediate state of axon damage that could persist for days in neuroinflammatory lesions. Second, this intermediate stage of damage can on the one hand progress to axonal fragmentation, but on the other hand also result in spontaneous recovery to a normal axonal shape. Further histopathological analysis of both EAE and MS tissue samples revealed that all stages of the FAD process also occur in myelinated axons, challenging the notion that demyelination is a prerequisite for the initiation of axon damage. Furthermore, by labelling both immune cells and axons, prolonged periods of contact especially of mononuclear phagocytes and axons could be captured, suggesting that these cells may play an important role in the induction of FAD (Nikić et al., 2011). However, phagocytes are not the only cell population that can interact with axons in neuroinflammatory lesions, as shown by Siffrin and colleagues using two-photon imaging in the brainstem of a pEAE model (Siffrin et al., 2010). In this model, neuroinflammatory lesions were induced by means of genetically labelled T-cells, which were $MOG_{35–55}$-specific and differentiated *in vitro* to Th17 cells. When transferred to

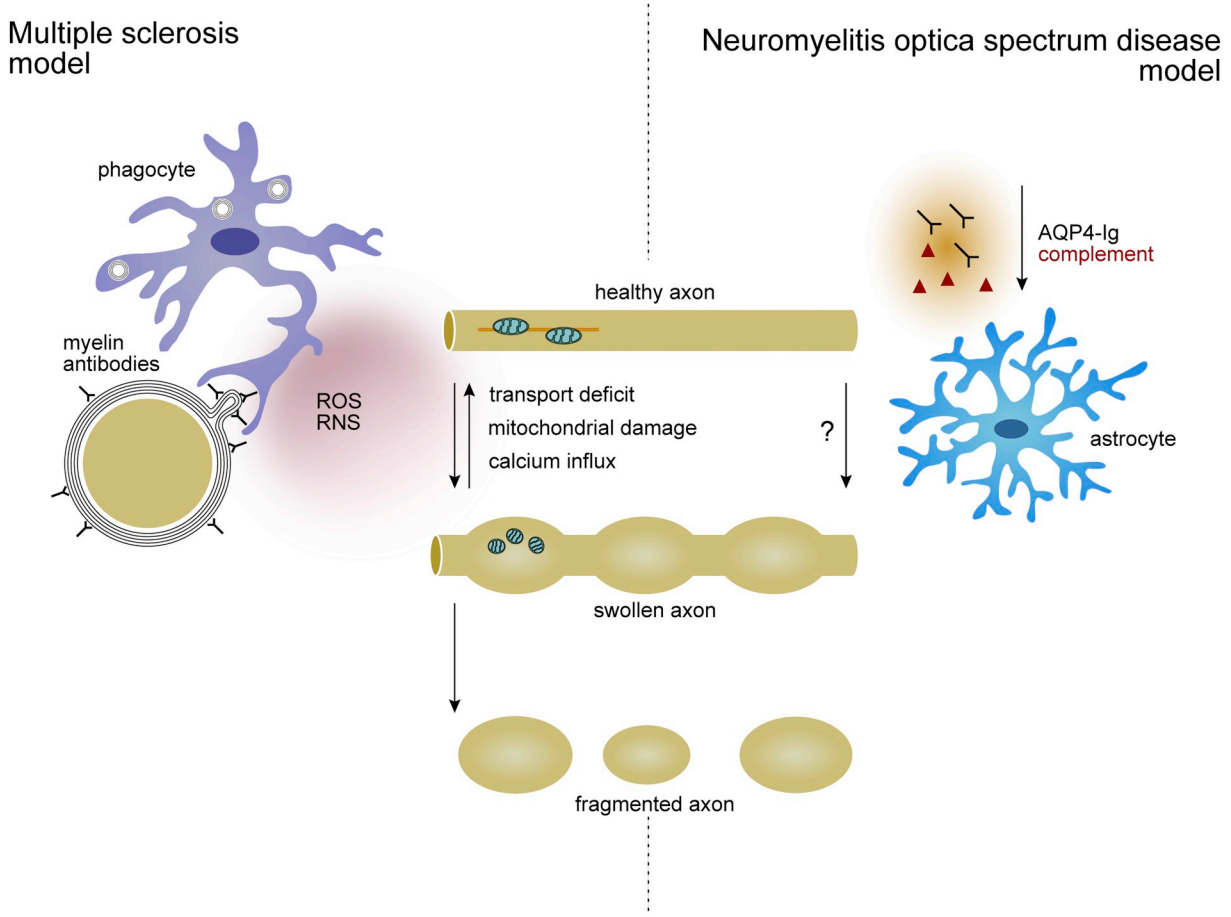

Fig. 1. Schematic of the execution phase of neuroinflammatory lesions in MS and NMOSD rodent models. As seen in intravital imaging, both neuroinflammatory models describe here lead to axonal swelling and glial pathology. While the basis of the axonal pathology and its late outcome in NMOSD has not yet been fully resolved, focal axonal degeneration in the MS model shows mitochondrial dysfunction, transport deficit and calcium influx as mediators.

lymphocyte-deficient Rag1$^{-/-}$ mice, these cells induced a severe non-remitting form of EAE. By visualizing neuronal cells in parallel (using *Thy1*:EGFP transgenic lines), the authors observed a close interaction of Th17 cells with neurons and their processes. As consequence of these interactions, an oscillating calcium increase was observed in neurons (using a genetically expressed calcium indicator in *Thy1*:CerTNL15 mice). This neuronal response was antigen-independent, and could be partially reverted by pharmacological inhibition of glutamate receptors (Siffrin et al., 2010). While these studies indicate that various immune cells can initiate axon damage in EAE, axon damage might also result from immune-initiated glial alterations, as demonstrated by Herwerth and colleagues in an NMSOD model. In this study, astrocyte depletion by application of AQP4-Ig and complement to the dorsal spinal cord rapidly induced axonal swellings. In contrast to FAD, where axonal fragmentation is a common occurrence, these axonal swellings, however, did not proceed to fragmentation within the next 6–8 h (Herwerth et al., 2016). This may indicate that immune mediators present in the MS models, but not in the acute antibody-mediated NMOSD model, are required for axonal transection and subsequent fragmentation (see Fig. 1).

One approach to reveal the molecular mediators of axonal fragmentation is to study the subcellular changes they induce in the affected axons. Here, our groups used a correlative light and electron microscopy (CLEM) approach to study axonal mitochondria in neuroinflammation. This approach allowed three-dimensional electron microscopic analysis of axons that were previously imaged *in vivo,* guided by laser-induced fiducial marks to target serial sectioning (Bishop et al., 2011). This analysis, which was corroborated by transgenic labeling, revealed altered mitochondrial morphology even in some normal-appearing axons, and in swollen axons that were still myelinated. These findings suggest that mitochondrial alterations are an early event on the path to axonal demise (Nikić et al., 2011). When imaging the mitochondrial membrane potential as a proxy of the energy household using tetramethylrhodamine methyl ester (TMRM), Sadeghian et al. could identify mitochondrial depolarization and dysfunction that was most pronounced in axons located in regions of extravasated inflammatory cells in the EAE spinal cord (Sadeghian et al., 2016). However, mitochondrial alterations in neuroinflammatory lesions not only affect their shape and function, but also their transport within spinal cord axons. By using double transgenic mice with both axonal and mitochondrial labelling, our groups found that transport of mitochondria along spinal cord axons was reduced both in swollen and in normal appearing axons within neuroinflammatory lesions (Sorbara et al., 2014). These pervasive transport deficits were already present in myelinated axons and led to an accumulation of mitochondria along axon segments within lesions. To explore, whether this local transport block was due a disruption of microtubules, which are the main tracks for mitochondrial transport, Sorbara et al. imaged mice that express a fluorescently labelled plus-end binding protein, EB3 (Kleele et al., 2014). Using this approach, the density and orientation of dynamic microtubules in axons in the spinal cord can be visualized. Interestingly, the detected drop in mitochondrial flux along the axon did not correlate to an apparent disruption of the microtubule cytoskeleton. This suggests that the observed mitochondrial transport deficits might have a signalling, rather than a structural cause, and could hence define an early and reversible axonal dysfunction – indeed, even

spontaneously, axonal transport deficits in EAE axons recovered during lesion resolution. Also, mitochondrial function recovered in EAE remission with complete clinical recovery of neurological function (Sadeghian et al., 2016).

When trying to further establish the causative cascade of subcellular damage mechanisms in neuroinflammation, causality can be difficult to establish given the multitude of proposed interactions between mitochondrial damage, reactive molecule species and ionic imbalances, such as calcium dyshomeostasis (Coleman, 2005; Friese et al., 2014; Mahad et al., 2009). One approach towards constraining this problem is the development of molecular *in vivo* imaging approaches that can reveal the temporal sequence of activation of these putative axon damage cascades. The required tools for such a 'multi-parametric' *in vivo* imaging approach are increasingly emerging (Breckwoldt et al., 2014; Grienberger and Konnerth, 2012; Sulis Sato et al., 2017). A number of studies have started to employ them in models of inflammatory (and non-inflammatory) axon injury. Taking advantage of the quantitative nature of fluorescence lifetime imaging (FLIM) and applying it to image autofluorescent molecules in living tissue (Elson et al., 2004), the groups of Niesner and Radbruch measured NAD(P)H levels in neuroinflammatory lesion in the brainstem *in vivo* (Mossakowski et al., 2015; Rinnenthal et al., 2013). This allowed distinguishing the free version from NADPH bound either to cellular housekeeping enzymes, or to NADPH oxidases (NOX), that directly produce of aggressive O_2^- radicals. In EAE lesions, a strong NOX activation was detected, which could be assigned to macrophages and microglia (Davalos et al., 2012), but also to astrocytes. The release of such reactive species could induce mitochondrial and axonal damage, as has been shown by direct application of reactive molecules to the exposed spinal cord (Nikić et al., 2011). At the same time, as shown in axotomy, mitochondrial damage can also be triggered by a rise in intra-axonal calcium (Breckwoldt et al., 2014) and indeed such intra-axonal calcium accumulations have been observed in axons in paradigms of neuroinflammatory and traumatic damage (Siffrin et al., 2010; Williams et al., 2014). Therefore, even though we have gained some insight regarding cause and effect of reactive molecules, calcium and energy household (see Fig. 1), the cascade of neuroinflammatory axon damage is still not fully delineated and needs to be assessed further, both in the acute and chronic situation.

7. Technical considerations when performing *in vivo* imaging in MS and NMOSD models

To successfully interrogate visualize the execution phase of neuroinflammation the imaging approach has to be tailored to anatomical location of CNS inflammation. As most EAE models result in strong immune infiltration of the spinal cord and in some cases the brain stem, these compartments are most often targeted for intravital imaging. And while spinal cord *in vivo* imaging originally used wide-field microscopy (Kerschensteiner et al., 2005; Vajkoczy et al., 2001), the complex cellular environment of inflammatory lesions typically requires the optical sectioning capabilities and scatter resilience of two-photon imaging. Imaging in spinal white matter tracts results in two major challenges that differ from the more established cortical imaging approaches: First, in contrast to imaging gray matter in the cortex, which by now can be completely penetrated by two-photon imaging in healthy mice (Ji et al., 2010; Kondo et al., 2017), inflamed white matter tracts pose substantial depth limitations. Indeed, several properties of healthy spinal cord, especially its high myelin content, degrade the laser focus (Helmchen and Denk, 2005) and limit imaging typically to the superficial ($< 100\,\mu m$) dorsal column. The immune response further reduces imaging quality, especially due to the presence of dense phagocytic cell infiltrates and oedematous dura thickening (Shrestha et al., 2017). Second, in contrast to the cortex *in vivo* imaging in mice, longitudinal imaging in the spinal cord is still in its infancy. While acute changes (*i.e.* those that unfold over $< 6\,h$) can be captured within one imaging session, chronic phenomena that take days or weeks to unfold are much harder to capture, as they require repeated imaging in surviving animals. Several studies have demonstrated the general feasibility of longitudinal imaging, either based on the repeated surgery (Davalos et al., 2008; Dray et al., 2009; Kerschensteiner et al., 2005; Lorenzana et al., 2015; Shi et al., 2011; Ylera et al., 2009) or the implantation of chronic spinal windows (Farrar et al., 2012; Farrar and Schaffer, 2014; Fenrich et al., 2012; Figley et al., 2013; Sekiguchi et al., 2016; Tang et al., 2015). While these techniques allow longitudinal observations of various aspects of inflammatory lesions, thus far most of them continue to be plagued by spurious inflammation and low success rates, making longitudinal observations – as now routinely possible in mouse cortex – an ongoing challenge and an area of need for future technology development. An elegant way to monitor CNS inflammatory activity longitudinally without invasive and repeated surgery his provided by ultra high-field MRI imaging of a marmoset model of MS (Lee et al., 2018; Maggi et al., 2014). Even though such 7 Tesla MRI has proven to be a powerful tool to visualize lesion evolution, its imaging resolution currently remains a limiting factor when assessing cellular responses.

8. Outlook

Despite the variety of *in vivo* imaging studies that have by now addressed the initiation and execution phases of neuroinflammatory white matter lesions, numerous questions remain unanswered for which a direct observational approach could be valuable. These will require pushing *in vivo* imaging further to add additional dimensions, for example concerning the biosensor modalities used, the anatomical areas assessed and the disease phases spanned by imaging.

One area of progress that is emerging, is to understand the metabolic consequences of a neuroinflammatory micromilieu for neurons and glia. The axon-glia unit not only enables efficient action potential conduction and synaptic transmission, but also forms an intricate network of metabolic interdependences, where processes as fundamental as cellular energy production and neurotransmitter synthesis are divided amongst interdependent cellular networks (Saab and Nave, 2017). The remodeling of neurons and glia, which results from repetitive and progressive inflammatory tissue damage, necessitates adaptations of these metabolic interactions that, while aiding short-term recovery, can later result in increased vulnerability or even progressive decline of the neural cells and circuits involved (Simons et al., 2014). Such adaptations thus represent a likely player in the secondary 'neurodegenerative' progression of MS, which remains refractory to most available MS drugs. As there has been substantial progress in developing novel genetically encoded metabolic sensors (GEMS) that allow quantitative metabolic imaging with subcellular resolution as reviewed in (Mohsin et al., 2015; Rogers and Church, 2016), *in vivo* imaging has the potential to contribute important insights into our understanding of metabolic (mal-) adaptations resulting from neuroinflammation. Such GEMS include, for instance, sensors for cytoplasmic and mitochondrial ATP levels or ATP/ADP ratios (Berg et al., 2009; Imamura et al., 2009; Tantama et al., 2013), other metabolic intermediates (Ewald et al., 2011; San Martin et al., 2013) or by-products of energy metabolism, such as reactive oxygen species (Zou et al., 2018). Importantly, it will likely become possible to measure corresponding metabolic signatures using NMR spectroscopy or PET to identify related metabolic signatures in the brain of MS patients (Ciccarelli et al., 2014), with the potential to corroborate findings from animal models or even devise biomarkers for progression.

Another area in need of more direct - ideally *in vivo* - study, is neuroinflammation-related damage of gray matter. Indeed, compared to white matter lesions, inflammatory processes in gray matter remain poorly described. While classically MS was considered a white matter disease, it is increasingly recognized that with disease progression pathological alterations also emerge in gray matter structures including the spinal cord gray matter, deep gray matter nuclei or cerebral cortex

(Calabrese et al., 2015; Trapp et al., 2018). Despite this emerging significance, we do not understand the cellular dynamics in such lesions well. On the one hand, this is due to the inability of two-photon imaging to reach the core spinal gray matter, and on the other hand due to a lack of suitable EAE models that mimic gray matter pathology.

To address the first limitation, the challenging optical environment of the heavily myelinated spinal dorsal column could be avoided. Indeed, pioneering studies in brain stem, cited above, have given important insights into the destructive interactions of immune cells and neuronal somata (Siffrin et al., 2010). In the spinal cord, several studies have demonstrated that targeting the dorsal horn rather than the dorsal column can provide access to gray matter. For example, Helmchen and colleagues used bolus loading with a small molecule indicator in the dorsal horn to perform first calcium imaging studies in spinal interneurons (Johannssen and Helmchen, 2010). A more recent study demonstrated the use of a one-photon miniaturized microscope mounted on a spinal cord imaging chamber to record neuronal and astrocytic calcium signals with cellular resolution in freely moving animals over several days (Sekiguchi et al., 2016). Moreover, a surgical approach to the ventral gray matter of the spinal cord has also been introduced (Cartarozzi et al., 2018). In rodent EAE models, this might allow assaying the remodeling of spinal circuits and the resulting changes in signal integration from sensory and descending inputs to motor neurons (Kerschensteiner et al., 2004). Alternative approaches for deep imaging in the brain, such as GRIND lenses (Barretto et al., 2009) or microprisms (Andermann et al., 2013; Chia and Levene, 2009), could also be adapted to spinal imaging. Whether such relatively invasive techniques will result in viable approaches without excessive tissue damage, inflammation or disruption of functional tracts remains unanswered.

Gray matter injury in MS is not limited to the spinal cord – thus future *in vivo* imaging studies will likely focus on more accessible sites of neuroinflammation. Recent studies of tissue derived from progressive MS patients have revealed a reduced number of dendritic spines in inflamed cortex and cerebellum (Albert et al., 2017; Jürgens et al., 2016). Notably, such spine loss was not restricted to inflamed areas, but also found in the surrounding normal appearing gray matter. This suggests that the CNS of MS patients is afflicted by widespread neuronal pathology beyond any focal lesions. This notion can be examined efficiently by *in vivo* imaging in EAE models, as both structural and functional imaging of cortical neurons is well established in mice (Grienberger and Konnerth, 2012; Holtmaat et al., 2009; Yang et al., 2010). Several studies have already investigated the cortical effects of systemic EAE which, however, does not result in substantial cortical inflammation. These studies have revealed increased spine turn-over, but stable spine numbers, during the initiation phase of EAE (Yang et al., 2013), as well as aberrant cortical hyperactivity in remission (Ellwardt et al., 2018), with both effects mediated by pro-inflammatory cytokines such as TNF-α. Moreover, imaging of intrinsic signals in systemic EAE revealed a shift in excitation-inhibition balance, in this case accompanied by increased spine densities (Potter et al., 2016). Together, these studies paint a complex picture of the remote effects of systemic neuroinflammation on cortical circuits – and raise the interesting question, how local neuroinflammation would affect cortical circuitry and function.

Search terms used for literature research

In vivo imaging multiple sclerosis/neuromyelitis optica model.
Multiphoton spinal cord multiple sclerosis/neuromyelitis optica.
Multiphoton multiple sclerosis/neuromyelitis optica.

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