Macroglial diversity and its effect on myelination
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Chapter 6

Summary, concluding remarks and future perspectives

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Summary

Macroglia in the central nervous system (CNS) consist of astrocytes (ASTRs) and oligodendroglial lineage cells. Oligodendrocytes (OLGs) mature from oligodendrocyte progenitor cells (OPCs) and are the myelinating cells of the CNS. Myelin, a stack of several lipid-bilayers wrapped around axons, facilitates saltatory conduction and provides metabolic axonal support. ASTRs support oligodendroglial lineage cell functioning and myelin membrane formation during development among others by the supply of fatty acids and cholesterol. Both myelin and OLGs are lost in multiple sclerosis (MS), resulting in demyelination accompanied by inflammation and neurodegeneration, which leads to neurological disability. The regeneration of new myelin sheaths, a process called remyelination, is essential for neuronal survival and recovery of neuronal function and halting further disease progression. ASTRs play a major role in orchestrating remyelination by transient signaling, among others, by extracellular matrix (ECM) remodelling. In MS, remyelination is often insufficient at a later phase during the disease. Currently, only disease-modifying immunosuppressive or immune-modulating treatments are available for MS.

Remyelination is more robust in grey matter (GM) areas than in white matter (WM) areas. This is observed both in MS as well as in experimental models of remyelination. This difference in regional remyelination efficiency may originate from intrinsic differences in OPCs differentiating into new OLGs and/or differences in extrinsic signals derived from ASTRs. Indeed, macroglia form diverse populations within the CNS, which will affect the response to injury and recovery, and contribute to pathology. Therefore, the aim of this work was to explore potential differences between macroglia in GM and WM and if so, whether and how these differences affect OPC behavior and in vitro remyelination. This knowledge may open therapeutic avenues to reinvigorate the endogenous remyelination process in MS and other demyelinating diseases.

In chapter 1, a comprehensive overview on the current knowledge on the diversity in macroglia from the GM and WM, as well as their interplay in health is given. In addition, the implications of regional diversity for remyelination and in light of its failure in MS are discussed. While OPCs appear transcriptionally relatively homogeneous, clear morphological and functional differences between gmOPCs and wmOPCs have been reported. Upon demyelinating injury, OPCs become heterogeneous at the transcriptional levels in experimental models, but in MS few transcriptional alterations are observed in OPCs. Mature OLGs do form a transcriptionally heterogeneous group of cells. The abundance of some mature OLG populations is reduced in MS, whereas others express immune-related proteins. While gmASTRs and wmASTRs are also morphologically, functionally and transcriptionally different, adult ASTRs appear less heterogeneous than mature OLGs on their transcriptional profile. ASTRs rather display a high functional plasticity and adapt to the specific local functional needs and injury responses, which differ between demyelinating injury in GM and WM. Therefore, macroglia heterogeneity and distinct local injury responses toward demyelination may add to the difference in remyelination efficiency in GM and WM, as observed experimental models and MS.

In MS lesions, the ECM protein fibronectin assembles into extracellular aggregates that impair remyelination. Chapter 2 addresses the underlying mechanism of fibronectin aggregate formation by ASTRs and potential differences in fibronectin aggregate formation between wmASTRs and gmASTRs. Using primary ASTRs, our findings revealed an initial activation in response to pro-inflammatory cytokines (first hit), which interferes with alternative fibronectin splicing, followed by a response to a Toll-like receptor 3 (TLR3) agonist (second hit) that decreases integrin affinity, resulting in release of fibronectin fibrils from the cell surface. While the order of magnitude of aggregation induced by TLR3 agonist Poly(I:C) was similar in gmASTRs and wmASTRs, the absolute levels of fibronectin aggregates formed were higher by wmASTRs than by gmASTRs. A similar diversity in alternative fibronectin splicing was observed between human post-mortem-derived control wmASTRs and MS wmASTRs. Thus, research into factors that interfere with alternative fibronectin splicing and TLR3-mediated signaling, and/or factors that prevent the decreased fibronectin-integrin binding aid to proper fibril formation, thereby precluding aggregation, are potential targets to aid remyelination in MS.
Cholesterol is an essential, major integral membrane lipid component of myelin, and is during development, and likely also upon remyelination, supplied by ASTRs to developing OLGs and incorporated into myelin membranes. In chapter 3 we aimed to address whether differences in remyelination in GM and WM may relate to differences in cholesterol supply by gmASTRs and wmASTRs. In vitro, wmASTRs and gmASTRs retained their morphological different phenotype (Fig. 1-1). We demonstrated that gmASTRs exported more cholesterol (Fig. 1-5) and were more supportive to in vitro myelination than wmASTRs (Fig. 1-2), but that specific inhibition of cholesterol biosynthesis in wmASTRs was beneficial for wmASTR-mediated modulation of in vitro myelination (Fig. 1-7). Reduced secretion of IL1β, likely by enhanced synthesis of non-sterol isoprenoids, and increased mRNA levels of Srebfic, a transcription factor involved in unsaturated fatty acid synthesis, both pathways upstream of committed cholesterol synthesis, masked the effect of reduced levels of wmASTR-derived cholesterol. Cholesterol efflux from either ASTR was reduced upon exposure to MS-relevant pro-inflammatory cytokines (Fig. 1-6), which was mediated via the cholesterol transporter ABCA1. Therefore, interference with non-sterol isoprenoid synthesis in ASTRs without interfering with cholesterol biosynthesis, may be a novel strategy to promote remyelination in WM MS lesions.

In both chapter 2 and 3, neonatal rat ASTRs were studied and the findings revealed that gmASTRs and wmASTRs were functionally diverse in modulating (re) myelination; with gmASTRs being more beneficial and wmASTRs more detrimental. In chapter 4 diversity of adult gmASTRs and wmASTRs was studied. Our findings demonstrated that cultured adult gmASTRs and adult wmASTRs were heterogeneous...
cells, showed diversity at the transcriptional level, in their ability to modulate *in vitro* myelination and in their response to TLR3 agonist Poly(I:C). More specifically, whereas wmASTRs were more reactive, and had higher amounts of transcripts relevant for ECM production and modification, gmASTRs more abundantly express genes involved in sterol biosynthesis. *In vitro* analysis demonstrated that wmASTR were less supportive for *in vitro* myelination likely by secretion of factors that perturb myelin membrane formation. Secreted factors from adult gmASTRs enhanced OLG metabolic activity and ECM coatings from gmASTRs enhanced OPC proliferation. When exposed to TLR4 agonist LPS, secreted factors from both gmASTRs and wmASTRs inhibited myelin membrane formation (Fig. 1-8), while TLR3 agonist Poly(I:C) elicited distinct responses. This diversity in gmASTRs and wmASTRs may contribute to more efficient remyelination in GM lesions than in WM lesions\(^{17–19,37,44}\).

In addition to ASTR diversity, diversity in OPCs may contribute to regional differences in remyelination. In *chapter 5*, potential differences between gmOPCs and wmOPCs were studied. Our findings showed that neonatal gmOPCs and wmOPCs displayed their own distinct properties *in vitro*, as reflected by differences in morphology, maturity and responses to environmental (injury) signals, including pro-inflammatory cytokines. Primary gmOPCs were morphological less complex and proliferated more in response to mitogens than wmOPCs, while wmOPCs were more mature at the gene expression level and differentiate faster than gmOPCs (Fig. 1-9). WmOPCs were more sensitive to IFNγ-mediated inhibition of proliferation and differentiation than gmOPCs, an effect that was potentiated by TNFα. Given that OPCs revert to a more immature state upon demyelination\(^{86}\), gmOPCs may have evolved to be better equipped for remyelination than wmOPCs, i.e. being more responsive to factors related to recruitment and less susceptible to inflammatory mediators, while the more matured stage provides wmOPCs an advantage in developmental myelination and myelin remodeling.

Taken together, the studies described in this thesis show that upon demyelination gmASTRs and gmOPCs may form an environment that is more permissive for remyelination than wmASTRs and wmOPCs. Of importance, many *in vitro* studies use macroglia derived from the cortex, a GM area, while experimental models for de- and remyelination often focus on white matter areas, such as the corpus callosum. This ignorance of macroglial diversity may lead to conflicting results obtained by *in vitro* and *in vivo* studies. Hence, for MS research and the development of a remyelination promoting therapy it is essential to consider differential effects in GM and WM lesions and that potential different strategies to promote remyelination in both regions may be required.
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Concluding remarks and future perspectives

The work described in this thesis provided insight on macroglia diversity in GM and WM and its relevance for \textit{in vitro} myelination and will assist in uncovering new therapeutic approaches aimed at enhancing endogenous remyelination in either area. A therapy that specifically aims to enhance remyelination in MS is currently lacking, although the anti-inflammatory activity of some medication used to treat relapsing-remitting MS, including fingolimod, dimethylfumarate and IFNβ, have been shown to act on ASTRs. In the following paragraphs, overall consequences of the findings described in this thesis for macrogia diversity on GM and WM remyelination, for inflammatory mediators on gmASTR and wmASTR behavior and for MS will be discussed, as well as future perspectives of therapeutic avenues for MS.

Consequences of grey white matter diversity for remyelination

Our findings show that regionally distinct ASTRs and oligodendroglial lineage cells likely contribute to the more robust remyelination in GM lesions than in WM lesions. For example, both neonatal and adult gmASTRs were more supportive towards \textit{in vitro} myelination efficiency than wmASTRs (chapter 3, 4). While wmASTRs produce more fibronectin aggregates that inhibit OPC differentiation (chapter 2), gmASTRs secrete more cholesterol, which enhances OPC differentiation (chapter 3). Moreover, adult wmASTRs appeared more reactive than gmASTRs and secreted soluble factors that inhibited myelin membrane formation (chapter 4). In addition, gmOPCs appear better equipped for remyelination due to their more immature phenotype, being more responsive to recruitment factors than wmOPCs (chapter 5), and increased differentiation towards secreted factors from neonatal gmASTRs (chapters 2, 5). On the other hand, wmOPCs were more susceptible to the detrimental effects of pro-inflammatory cytokines than gmOPCs (chapter 2), while wmOPC differentiation was not enhanced in response to secreted factors from wmACM (chapter 4). Hence gmASTRs and gmOPCs may together provide an environment more permissive towards remyelination than wmASTRs and wmOPCs. Therefore, elucidation of which secreted factors from ASTRs promote or inhibit OPC differentiation and/or myelin membrane formation will lead to identification of putative new targets to enhance remyelination. For example, these may include 8,9-unsaturated sterols, fatty acids, which have been shown to enhance OPC differentiation and remyelination. Our unpublished data uncovered with the use of fractionation of astrocyte-conditioned medium that both gmASTRs and wmASTRs secrete a soluble factor larger than 30 kDa that inhibits myelin membrane formation. On the other hand, the extent of OPC differentiation in MS lesions may not solely rely on the type of OPC, the amount of cholesterol effluxed from ASTRs, nor the presence of fibronectin aggregates, but also depend on the presence and availability of local injury signals which are different in GM and WM lesions.

Consequences of inflammation for grey and white matter astrocyte modulation of myelination

Intrinsic differences between gmASTRs and wmASTRs may differently modulate remyelination by responding in a region-specific manner to demyelinating injury dependent on the local inflammatory environment. This inflammatory environment may include (1) TLR3 agonists, such as stathmin present in myelin debris, which induced fibronectin aggregate formation by neonatal ASTRs (chapter 2), (2) TLR4 agonists, such as Hsp70 or Tenascin-C in vivo, which induced the secretion of soluble factors by adult ASTRs that inhibit myelin membrane formation by differentiated wmOPCs (chapter 4), and/or (3) pro-inflammatory cytokines TNFα, IFNγ and IL1β, which alter fibronectin splicing thereby potentiation the aggregation potential of the molecule (chapter 2), decreased ABCA1 expression in gmASTRs and reduced cholesterol efflux from both gmASTRs and wmASTRs, and decreased genes encoding for enzymes involved in unsaturated fatty acids biosynthesis in gmASTRs (chapter 3). Notably, more myelin debris is present in WM lesions, and inflammation is more pronounced in WM MS lesions compared to GM MS lesions. Hence, myelin debris and inflammation may play a more prominent role in MS pathology of WM lesions. Moreover, wmASTRs were by default more reactive, both \textit{in vitro} (chapter 4) and \textit{in vivo}, and expressed genes that relate to a neurotoxic A-ASTR subtype (chapter 4) more abundantly, which are also present in WM MS lesions. A2-ASTRs, considered to be more neuroprotective, have not been studied in the context of MS yet. As our findings demonstrate that more transcripts related to A2-ASTRs were present in adult gmASTRs and gmASTRs were more supportive for myelination (chapter 4), it would be interesting to investigate whether A2-ASTRs may be present in GM MS lesions, are indeed beneficial for myelination and...
Consequences for multiple sclerosis

Most of the studies described in this thesis are performed with rat-derived ASTRs. As human ASTRs are up to three-fold larger and morphologically more diverse, it remains to be determined whether these findings are directly transferable to MS. Our studies show that human post-mortem-derived MS wmASTRs form remyelination-inhibiting fibronectin aggregates in vitro in the absence of cytokines and TLR3 agonists (Chapter 2). The ability of MS wmASTRs to form fibronectin aggregates in vitro may depend on inflammatory mediators they have encountered in vivo. These may include pro-inflammatory cytokines and endogenous available TLR3 agonists such as stathmin. Alternatively, MS ASTRs may intrinsically be more prone to form aggregates. However, this is difficult to determine as ASTRs obtained from post-mortem MS brain tissue have encountered a diseased environment. To address this, ASTRs generated from induced pluripotent stem cells (iASTRs) obtained from MS patients and healthy subjects, could be used as a model to study fibronectin aggregation in injury-naïve normal and MS iASTRs. Rat-derived gmASTRs form less fibronectin aggregates than wmASTRs (Chapter 2), but the presence of (aggregated) fibronectin in GM MS lesions is not well-studied yet. As the lesion environment between GM and WM lesions is different with regard to their inflammatory profile, studying fibronectin splicing and aggregation in GM gmASTRs will be of relevance. A recent study describes the generation of distinct GM-like and WM-like iASTRs. Hence, also here iASTRs will be of use to study fibronectin aggregation by naïve MS gmASTRs. Preventing fibronectin aggregate formation by interfering with fibronectin splicing and TLR3-mediated signaling may prove beneficial as an approach for enhancing remyelination in MS. On the other hand, if MS ASTRs are intrinsically more prone to form aggregates, means to degrade fibronectin with for example metalloproteinase 7 (MMP7), or means to overcome inhibition of remyelination in the presence of fibronectin with for example ganglioside GD1a, may prove beneficial for MS.

Therapeutic avenues for multiple sclerosis

Our findings showed that selective interference with lipid biosynthesis in ASTRs, i.e., cholesterol, unsaturated fatty acids and non-sterol isoprenoids may be a novel strategy to promote remyelination in WM MS lesions. Both in MS and EAE, an animal model for MS, genes encoding for enzymes involved in cholesterol biosynthesis, including Hmcgs1, Fdps and Fdft1, are downregulated in ASTRs. Of interest, statins, which are prescribed as cholesterol lowering drugs, have shown to be beneficial for MS, by an immuno-regulatory effect likely via a decreased isoprenylation in peripheral immune cells, but may actually be detrimental for OPC differentiation and remyelination due to a decrease in biosynthesis of cholesterol, an essential component of myelin, and 8,9-unsaturated sterol cholesterol precursors. Hence, therapeutics that aim to control inflammatory response specifically in ASTRs via enhancement of isoprenylation without affecting cholesterol efflux and lipid biogenesis may therefore benefit remyelination in MS. The interaction between the secretion of inflammatory mediators, cholesterol transport and unsaturated fatty acid synthesis is tightly regulated. Inflammatory mediators are less present in GM MS lesions compared to WM MS lesions, and consequently may support the efflux of cholesterol more. In chapter 3, the main lipid studied was cholesterol. However, chapter 3 revealed that next to cholesterol, unsaturated fatty acids and non-sterol isoprenoids, by means of isoprenylation of signaling molecules, likely also play an important role in ASTR-mediated support of myelination. Cholesterol and other lipids can be released from ASTRs within different particles; high- and low-density lipoprotein (HDL and LDL) particles contain high levels of cholesterol, but also exosomes have an enhanced cholesterol content compared to the cell plasma membrane. Hence, exosomes are extracellular vesicles released from multi-vesicular bodies that fuse with the plasma membrane. Of special interest is that LDL particles induce a pro-inflammatory response, while HDL particles induce an anti-inflammatory response. An in-depth study of the type lipid-particles released by ASTRs, as well as the support of HDL particle formation and secretion could reveal new information on the supply of ASTR components to OPCs and OLs.
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It has been suggested that MS is not a disease of the immune system, nor a primary neurodegenerating disease, but actually a defect of the lipid metabolism. According to Corthals\textsuperscript{493} MS is comparable to atherosclerosis; a dysregulation of the lipid metabolism which in arteries causes atherosclerosis. In the brain, a dysregulation of the lipid metabolism could contribute to MS, but the underlying etiology would be the same as in atherosclerosis. In favor with this reasoning, the lipid composition in myelin of MS normal appearing white matter (NAWM) is changed compared to control myelin\textsuperscript{494}, and an altered lipid composition is observed in cerebrospinal fluid of MS patients compared to control\textsuperscript{495}. Both lipid homeostasis (chapter 3 and 4) as well as fibrotic scarring (chapter 2 and 4) play a role in atherosclerosis, as in MS. One of the major risk factors for a disruption of lipid homeostasis is LDL cholesterol, which correlates with an increased dietary intake of animal fats and sugar. Under physiologically conditions, cholesterol and other lipids are locally produced within the brain mostly by ASTRs and not taken up from the periphery into the brain\textsuperscript{377}. The removal of myelin debris is essential for remyelination and is initiated by ASTRs\textsuperscript{15}. However, similar as in atherosclerosis, an overload of myelin debris-derived cholesterol forms crystals in myeloid cells in the brain which hampers remyelination. An increase of cholesterol efflux out of the cells is required to resolve these cholesterol crystals and for remyelination to take place\textsuperscript{378}. Of importance, cholesterol efflux in ASTRs is inhibited by pro-inflammatory cytokines (chapter 3). Hence, resolution of inflammation may be required for sufficient cholesterol efflux from both ASTRs and myeloid cells in MS. On the other hand, in an experimental model of demyelination in which the blood-brain-barrier is compromised, lipids including cholesterol and unsaturated fatty acids are imported into the brain via dietary uptake and contribute to remyelination by OLGs\textsuperscript{372,373}, thus being less dependent on ASTR-derived cholesterol. Hence, observing MS as a disease of lipid homeostasis comparable to atherosclerosis may yield new insights into the mechanism underlying MS remyelination pathology. Taken together, the development of a therapy for MS, which enhances isoprenylation and unsaturated fatty acid production, without affecting cholesterol biosynthesis and secretion, may prove beneficial for MS.