Macrogliov diversity and its effect on myelination
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Chapter 1

The white and grey areas of macroglial diversity and its relevance for remyelination (failure)

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Abstract

Macroglia, comprising astrocytes and oligodendroglial lineage cells, have long been regarded as uniform cell types of the central nervous system (CNS). Although regional morphological differences between these cell types were initially described not long after their discovery almost a century ago, these differences were largely ignored. Recently, accumulating evidence suggests that macroglial cells form diverse populations throughout the CNS based on both functional and morphological features. Moreover, with the use of refined techniques including single-cell and single-nucleus RNA sequencing, additional evidence is provided for regional macroglia heterogeneity at the transcriptional level. In parallel, several studies have shown regional differences in remyelination capacity in CNS grey versus the white matter areas, both in experimental models for successful remyelination as well as in the chronic demyelinating disease multiple sclerosis (MS). In this review, we provide an overview of the diversity in oligodendroglia lineage cells and astrocytes from the grey and white matter, as well as their interplay in health and upon demyelination and successful remyelination. In addition, we discuss the implications of regional diversity for remyelination and in light of its failure in MS. Although a plethora of differences in local macroglia are discussed here, it is currently difficult to discern how their interaction contributes to differences in local remyelination capacity and MS pathology, as the local inflammatory injury signals differ between grey and white matter and thereby affect macroglial identity. Since the etiology of MS remains unknown and only disease-modifying treatments altering the immune response are available for MS, the elucidation of grey versus white matter macroglial diversity and its putative contribution to the observed difference in regional remyelination efficiency may open up therapeutic avenues aimed at enhancing endogenous remyelination in either area.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) characterized by inflammation\(^a\), astrogliosis\(^b\), and neurodegeneration\(^c\)–\(^d\). MS is a heterogeneous disease both at the clinical and pathological level. More specifically, MS can manifest in different disease courses, most commonly starting with relapsing-remitting MS (RRMS) characterized by inflammation-mediated exacerbations related to acute demyelination in the CNS, and subsequent recovery. MS may also present in a progressive form in the absence of remission, either initially as in primary progressive MS (PPMS), or following its relapsing form, called secondary progressive MS (SPMS). Neurodegeneration, caused among others by ultimate failure of remyelination is, amongst others, an underlying cause of disease progression\(^e\)–\(^f\). Treatments for MS are limited to disease modifying treatments that reduce inflammation, while a regenerative treatment overcoming the failure of remyelination is currently unavailable. Of specific interest is that MS heterogeneity is also reflected in differences in pathology in different CNS regions, which is best studied in leukocortical lesions that span both grey matter (GM) and white matter (WM). For example, in leukocortical lesions, remyelination is more robust in the GM part than in its WM counterpart\(^g\)–\(^h\), while also differences in cellular density and activation are present\(^i\). This regional diversity in cellular identity and/or responses may underlie differences in regional remyelination, and although these lesions may remyelinate, remyelination is often insufficient in either area\(^i\).

The CNS consists of neurons, microglia and macroglia, the latter comprising astrocytes (ASTRs) and oligodendroglia, i.e., myelin-forming oligodendrocytes (OLGs) and OLG progenitor cells (OPCs). In the adult human brain, the ratio of glial cells to neurons is \(\sim 1:1\) or even smaller, unlike a \(\sim 10:1\) ratio, as reported in previous literature\(^i\)–\(^j\). The CNS can be grossly divided in two regions, the GM and the WM. The GM contains mainly neuronal cell bodies, dendrites and axon terminals, whereas axons primarily reside in the WM. Thus, synapses are more prominent in GM areas, while WM has a higher myelin content. Also, the abundance of oligodendroglia and ASTRs in the CNS is not uniform and is region dependent. In most adult human brain regions, OLGs are the most numerous of glial cells, with a percentage ranging from 20% in the visual cortex\(^k\)–\(^l\), to 75% in the neocortex\(^m\)–\(^n\). In rodents, OPC numbers vary from 3% in GM to 8% in WM\(^o\). Also when comparing human normal appearing GM and WM,
OPCs are more abundant in WM (98/mm$^2$ versus 140-150/mm$^2$)$^{37,60}$. While numerous in the WM part of the frontal cortex (69%), only 36.6% of glial cells are OLGs in its GM part$^{36,64}$. ASTRs follow OLGs in numbers in most brain areas, including in the frontal cortex WM at 24% of glial cells, but not in the frontal cortex GM, where they outnumber OLGs at 46.5% of glial cells$^{36,64}$. Over the past years, evidence has been accumulating indicating that macroglia from the GM and WM display regional plasticity and intrinsic heterogeneity, the first being adaptations of the same cell type to the local functional needs and responses to injury, and the latter being intrinsic transcriptional differences in cell populations. These regional differences will have consequences for cell functioning upon CNS injury, such as demyelination and remyelination. Indeed, similar as observed in leuckocortical MS lesions, in the cortex, a GM area, remyelination is more efficient in experimental models for successful remyelination than in the corpus callosum, a WM area$^{70,84}$. Here, we review current literature on diversity of macroglial cells, and discuss how this may contribute to regional differences in successful remyelination and upon remyelination failure. We will start with an introduction into macroglia, followed by a detailed overview on the topic of oligodendrogial and astroglial diversity in health, focusing on GM and WM (summarized in Figs. 1,2). Next, we discuss macroglia diversity in the context of regional differences in successful remyelination, and in light of remyelination failure and its implications for MS (summarized in Figs. 2,3). Hence, this review proposes to take regional differences into account when developing and/or assessing remyelination-based treatments for MS.

**Introduction to macroglia**

**Oligodendroglial cells**

OLGs ensheathe axons with myelin, which is a tight stack of several lipid bilayers that provides metabolic support to axons$^3$ and facilitates rapid saltatory conduction of nerve impulses$^{37,65}$. In addition, OLG lineage cells are involved in synapse modulation and neurotransmission in both GM and WM$^{36,65}$. OLG lineage markers include the transcription factors oligodendrocyte transcription factor 2 (OLIG2) and SRY-box transcription factor 10 (SOX10). Mature OLGs develop from OPCs, which are platelet-derived growth factor receptor α (PDGFRα) and chondroitin sulphate proteoglycan 4 (CSPG4 in human, also known as neuron-glial antigen 2 (NG2) in rodents) expressing cells that comprise ~5% of the adult rodent CNS$^{66-68}$. Of note, PDGFRα and NG2 are co-expressed on >99.5% of non-vascular cells in the CNS$^{68-69}$. Upon maturation, the cells pass an immature, pre-myelinating state in which they express stage-specific markers including breast carcinoma amplified sequence 1 (BCAS1) and ectonucleotide pyrophosphatase/phosphodiesterase 6 (ENPP6)$^{70-72}$, while the myelin-typical lipids sulfatide and galactosylceramide appear at the cell surface. Myelinating OLGs are recognized by their expression of myelin-specific proteins of which myelin basic protein (MBP) and proteolipid protein (PLP) are the major ones$^{73}$.

In rodents, the process of developmental oligodendrogenesis and subsequent myelination is well-studied. Using fate mapping, Kessaris and colleagues elegantly showed that OPCs are derived from radial glia and populate the murine brain in 3 waves. At embryonic day 11.5 (E11.5) a first wave of OPCs emerges from the medial ganglionic eminence and anterior entopeduncular area. A second wave is generated from the lateral and/or caudal ganglionic eminences at E15. The OPCs that emerge from both waves populate the murine telencephalon in a ventral to dorsal manner. The third wave of OPCs occurs in the first week after birth and originates from the cortex. Interestingly, OPCs that are derived from the first wave of OPCs disappear after birth and are virtually undetectable in adulthood$^{75}$. Also, the highly-orchestrated process of developmental myelination is well-studied. First, OPCs proliferate$^{76}$ and migrate to the axons to be myelinated$^{77}$. There OPCs differentiate into pre-myelinating OLGs and extend multiple processes that contact axons but do not yet myelinate. Upon repeal of mainly axon-derived inhibitory factors for OLG differentiation (reviewed in$^{78}$), pre-myelinating OLGs retract their secondary and tertiary processes and start synthesizing considerable amounts of myelin-specific proteins, including MBP and PLP, and myelin-typical lipids, including galactosylceramide, sulfatide and cholesterol, that are required to form the compacted myelin segments at their primary processes which enwrap the receptive axon$^{79}$.

PDGFRα immunolabelling revealed that OPCs are more abundant in the corpus callosum (~120 cells/mm$^2$, or 8% of cells) compared to the cortex (~80 cells/mm$^2$, or 3% of cells) of young adult mice$^{80}$. Each OPC occupies an individual niche that
Astrocytes

ASTRs have a plethora of functions, including providing trophic support to neurons, regulating synapse formation and pruning, maintaining the integrity of the blood-brain-barrier (BBB)\(^{86-90}\), and support of OLGs during developmental myelination\(^{16,91}\). In rodents, the first ASTRs are detected at E16, just before the first OPCs are formed. Like OPCs, the vast majority of ASTRs are formed during the first month after birth, i.e., the ASTR population increases 6-8 fold\(^{91,95}\). During development, most ASTRs derive after the formation of first neurons and OPCs, out of their common neural progenitors called radial glia\(^{64-66,85}\). Radial glia are a heterogeneous population of cells which is formed based on a spatial and temporal patterning program in a columnar organization\(^{15,44,97}\). Whereas OPCs are derived mostly from the motor neuron progenitor (pMN) domain, ASTRs maintain the columnar organization formed by the radial glia\(^{65,64,66,95}\). ASTRs do not derive from the pMN domain, but from three other progenitor domains named p1, p2 and p3, with p1 being the most dorsal and p3 being the most ventral domain\(^{95}\). After asymmetrical migration of newly formed ASTRs, ASTRs locally proliferate symmetrically and hereby largely increase the number of ASTRs in the brain\(^{91,96}\). The final ASTR phenotype is thought to depend on its local cellular environment as well as on the region-specific functional demands\(^{15,44,94}\). Markers of immature ASTRs include Fabp7/Blbp and Fgfr3\(^{44,66-70}\). Mature ASTR markers include Aldh1l1, S100b, Aldoc, Acsgb1, and Pla2\(^{44,96}\), but there is no marker which labels all ASTRs. The absence of a uniform ASTR surface marker frustrates the isolation of single ASTRs. Astrocytogenesis is promoted by Sox9 and Nifa/b\(^{43,44,94}\), with Sox9 being especially important for gmASTRs development\(^{39}\). This suggests that Sox9 may have a possible role in ASTR diversification\(^{39,100}\). ASTRs are further characterized by the presence of filamentous proteins, including vimentin, desmin, synemin and glial fibrillary acidic protein (GFAP)\(^{84-87}\), of which GFAP is the most abundant intermediate filament expressed in ASTRs\(^{86-89}\). Around postnatal day 14-21 ASTRs are considered to be morphologically mature\(^{89}\) and further aging of murine ASTRs does not induce major changes into their homeostatic and neurotransmission-regulating genes\(^{86-88}\). In addition, it has been suggested that ASTRs go into senescence\(^{90}\), and aged murine ASTRs upregulate genes involved in synapse maturation elimination and down-regulate genes related to mitochondrial function and anti-oxidant capacity\(^{90}\). Moreover, aging of ASTRs does induce the formation of a more pro-inflammatory ASTR phenotype\(^{90,91}\).

In conclusion, macroglia develop sequentially from radial glia during development, and obtain age-related changes in their phenotype and transcriptional profile. In addition, in recent years evidence has accumulated that regional macroglia appear as diverse populations throughout the CNS. In the following section, current knowledge on the regional diversity of OPCs, OLGs and ASTRs in GM and WM areas of healthy CNS will be outlined (summarized in Figs. 1,2).

Oligodendroglial diversity

Heterogeneity of OPCs in the grey and white matter

A transplantation study by Viganò and colleagues\(^{95}\) hinted at regional differences between OPCs derived from the GM and WM. It was found that wMOPCs differentiate into OLGs equally well in both healthy GM and WM, whereas gMOPCs remain more immature, irrespective of the environment. Hence, OPCs seem to carry a memory or intrinsic potential that is not altered by a new and different environment. In other words, gMOPCs and wMOPCs have different phenotypes which may be functionally different as well\(^{95}\). Indeed, OPCs have been described to show diversity in electrical properties\(^{84,85,97}\), gene expression profiles \(in vitro\)\(^{98-99}\), proliferation\(^{14,15,16}\) and differentiation\(^{99,101-103}\) rates, injury responses\(^{100,103-104}\), or otherwise\(^{63,105-107}\). Already...
in 2002 it was reported that proteolipid protein (PLP/DM20) mutations affect OPC production more in the cortex than in the corpus callosum, indicating that oligodendrogenesis is differentially regulated between GM and WM\(^{46}\). Subsequent studies in rodent models show that in vivo, wmOPCs mature more efficiently into myelinating OLGs than gmOPCs, which proliferate slower and produce fewer mature cells while cell survival is comparable\(^{60,81,122,129-131}\). Possibly as a consequence of this, OPC density in the adult rodent brain is higher in WM (8%) than in GM (3%)\(^{60}\) (Fig. 1). Of interest, the amount of proliferative gmOPCs declines with age while the proportion of wmOPCs that proliferates remains stable\(^{69}\). However, upon aging, the percentage of proliferative OPCs becomes similar in both GM and WM\(^{85}\). Additionally, gmOPCs repopulate less than their WM counterparts, which are fully repopulated upon being depleted when Smoothened, a regulator of sonic hedgehog (Shh) signaling, is conditionally deleted during development. This implies that gmOPCs are more dependent on Shh signaling for expansion\(^{36}\). In vitro, rodent neonatal gmOPCs are morphologically less complex, express less of common OLG-maturation genes, proliferate more and differentiate slower than wmOPCs\(^{121}\) (Fig. 1). Regulation of proliferation depends on the mitogen, as wmOPCs proliferate more in response to PDGF than gmOPCs\(^{133}\). These findings indicate that wmOPCs are more mature than gmOPCs, even after prolonged culture in vitro\(^{121,122}\) (Fig. 1). That OLG lineage cells in the WM show a more complex phenotype in vitro is supported by an in vivo study describing that premyelinating wmOLGs in the corpus callosum have more processes and myelinate more axons in the developing rat brain at postnatal day 7 than premyelinating gmOLGs in the cortex\(^{134}\). Furthermore, in the rat cortex at postnatal day 50, NG2-positive OPCs present in a classical stellate form with processes radiating in all directions. By contrast, in the corpus callosum OPCs show an elongated morphology with multiple processes that follow axons. Additionally, OPCs in the rat corpus callosum produce longer processes than OPCs in the cortex\(^{59}\). In line with this, in the adult human brain, gmOPCs have a more regular network-like appearance than wmOPCs\(^{60}\). Other studies report differences in voltage-gated ion channels and spiking behavior of gmOPCs and wmOPCs\(^{60}\). More specifically, OPCs from the cortex have higher densities of AMPA/kainate receptors, while OPCs from the corpus callosum have higher densities of NMDA receptors at P9 (Fig. 1). This observation may underlie the observed differences in regional proliferation and differentiation rate. Thus, the shorter cell cycle time of wmOPCs may be explained by a higher density of voltage-gated potassium channels and subsequent higher peak outward current in WM\(^{46,57}\), as electrical activity is known to stimulate OPC proliferation either by stimulating the release of PDGF from neurons or making wmOPCs more responsive to PDGF\(^{56}\). In turn, as NMDA receptors are involved in...
activity dependent myelination\textsuperscript{70,144}, the higher expression of NMDA receptors on wmOPCs may contribute to the greater differentiation potential of wmOPCs\textsuperscript{85}.

As the expression of receptors on OPCs differs and as OPC proliferation and differentiation are influenced by extrinsic differences, environmental cues may contribute to differences in OPC diversity. For example, in the GM there are more environmental signals, though it is unspecified where the signals that decrease OPC proliferation and arrest differentiation into OLGs more than in the WM are derived from\textsuperscript{85,121,144}. When developing rats are exposed to cuprizone, a toxic copper chelator depleting OLGs, via a maternal diet from gestational day 6 to postnatal day 21, the density of OLG lineage cells is widely impaired in the cortical regions at postnatal day 21, whereas only mature OLGs are affected in the corpus callosum\textsuperscript{85}. An increased expression of the anti-aging protein Klotho may protect wmOPCs from cuprizone intoxication\textsuperscript{145}. On the other hand, while prenatal PDGFRα-positive OPCs display remarkable regional heterogeneity at the transcriptional level in mice, the transcription differences converge to a common region-independent profile upon transition to neonatal OPCs\textsuperscript{144}. Single-cell RNA sequencing (scRNAseq) on murine CNS tissue from various brain regions from the developing and young adult murine brain revealed also a single OPC population independent of region or age\textsuperscript{144} (Fig. 2). However, OPCs in the developing brain show more transcriptional signs of proliferation than OPCs in the more mature brain. In addition, a differentiation-committed OPC (COP) population was identified which appears slightly more abundant in the WM corpus callosum than in the GM somatosensory cortex\textsuperscript{85,123}, and may reflect a difference in maturation state of the region in the developing brain. Similarly, independent single-nucleus RNA sequencing (snRNAseq) studies on post-mortem human brain tissue identified only one OPC population\textsuperscript{144,145}. Hence, although it has been suggested that OPCs arising from the different waves might be functionally different and myelinate specific brain regions\textsuperscript{144,145}, in the developing CNS PDGFRα-positive pre-OPCs converge on a transcriptional level, i.e., postnatal OPCs from brain and spinal cord present an almost similar transcriptional profile\textsuperscript{145}. However, at postnatal day 7 OPCs from the spinal cord are more mature than OPCs in the brain based on the expression of late-stage differentiation markers (Mog/Mag/ Mal)\textsuperscript{144}. Also, in favor of a single OPC population is that OPCs derived from the three different waves initially present comparable electrophysiological capacities. However, the authors show that after the first postnatal week OPCs become regionally diverse in ion channel expression, i.e., cortical OPCs show a higher AMPA/kainate receptor density and OPCs from corpus callosum a higher NMDA receptor density\textsuperscript{85}. This indicates that PDGFRα-positive pre-OPCs reprogram their transcriptional system during development\textsuperscript{144}. Overall, OPCs from different regions are transcriptionally similar, and given their limited motility rather acquire differences in protein expression and function during maturation into mature OLGs via their local micro-environment.

**Heterogeneity of oligodendrocytes in the grey and white matter**

In the rodent CNS, OPCs differentiate into myelinating OLGs up to 8 months after birth\textsuperscript{60,81,122}. This differentiation can be initiated by, and is required for, the learning of complex tasks\textsuperscript{146,147}. In humans, OLGs may be produced continuously although their proliferation declines with age\textsuperscript{85,150,151}. Like in rodents, the learning of a complex motor task induces myelination remodeling in human OLGs\textsuperscript{148}. OLGs have the highest oxidative metabolism of all cells in the CNS during active myelination\textsuperscript{144} for the production of a high amount of membranes that can take up to 100 times the weight of the cell\textsuperscript{85}. Additionally, levels of the anti-oxidant glutathione are remarkably low in OLGs\textsuperscript{85}. These features might explain why myelinating OLGs are exceptionally vulnerable to metabolic stress\textsuperscript{145}, possibly contributing to the multitude of pathologies involving demyelination. In mice, gmOLGs show less morphological plasticity. More specifically, two very recent in vivo live imaging studies\textsuperscript{152,153} show that cortical OLGs hardly remodel their internodes while WM internodes are thickened upon increased axonal activity\textsuperscript{152} or can be elongated when a neighboring internode is ablated in zebrafish\textsuperscript{153}. In the human WM, OLG turnover is especially low and most OLGs are formed in the first decade of life with an annual turnover of ~1 in 300 OLGs (0.3%). This in contrast to adult human GM, where the expansion phase of OLGs appears to be much longer, up to the fourth decade of life; combined with an annual turnover of 2.5\%\textsuperscript{152}. Whether diversity of OLG phenotype can be branded as heterogeneity of OLG lineage cells or their plasticity is recently reviewed by Foerster, Hill & Franklin\textsuperscript{85}.

Heterogeneity of mature OLGs was first observed in the 1920s by Pio del Rio-Hortega. Based on morphology he described OLGs with small cell bodies and...
many fine processes that reside both GM and WM, and three additional distinct subtypes that are restricted to the WM\textsuperscript{56,57}. After this initial observation of the four morphological distinct mature OLG subpopulations, OLG heterogeneity was mostly ignored and only recently more attention has been drawn to heterogeneity of OLGs\textsuperscript{49}. The rise of sequencing techniques allows the study of transcriptomics and has provided a considerable contribution to the knowledge of regional heterogeneity of developing OLGs in the last years\textsuperscript{58}. First, Zhang and colleagues produced a detailed comparison of the transcriptome of the different cell types of the mouse cortex, including three oligodendroglial maturation stages\textsuperscript{39}. Zeisel and colleagues performed quantitative single-cell analysis of the transcriptome on cells of the mouse primary somatosensory cortex and the hippocampal CA1 region\textsuperscript{40}. This study demonstrated the possible existence of six OLG subpopulations based on gene expression may represent different maturation stages of which one appears specific to the somatosensory cortex\textsuperscript{45,46}. scRNAseq on PDGFRα-derived oligodendroglial cell types from various brain regions of the developing and young adult murine CNS categorizes 12 OLG lineage populations that include five different maturation stages, including 1 murine OPC stage (mOPC), 1 murine differentiation-committed (mCOP) stage, 2 murine newly-formed OLG stages (mNFOl), 2 murine myelin-forming OLG stages (mMFOl) and 6 murine mature OLG (mMOL) stages (Fig. 2a). Of interest, of the six mMOL stages, mMOL3-4 were enriched in myelination genes and genes involved in lipid biosynthesis, while transcripts for synapse genes are enriched in mMOL5-6 (Fig. 2a), which are both predominantly present in the adult brain. In contrast to mOPCs, which are transcriptionally similar between brain regions, the mMOL5 population is enriched in the somatosensory GM cortex, and mMOL1, 4, 5 and 6 are enriched in the WM corpus callosum compared to other brain regions\textsuperscript{42}. The identification of six different mMOL stages confirms heterogeneity of mature OLGs at the transcriptional level and their transcriptional profile indicates regional heterogeneity in mMOL function, including genes related to synaptic functions instead of myelination in the GM cortex. Regional mature OLG heterogeneity may be acquired by the micro-environment upon differentiation-inducing cues\textsuperscript{40}, which is also previously described in human development\textsuperscript{43,46}.

Similarly, using snRNAseq, six groups of mature OLGs in the adult human brain WM can be distinguished, Oligo1 to Oligo6\textsuperscript{42}. As some shared similarities with mMOL groups defined in mice are evident\textsuperscript{42}, mature human OLGs populations are from here on referred to as hMOL1 to hMOL6. Pseudo-time analysis revealed two major developmental end-stages of hMOLs; hMOL6 developed via hMOL4 into an end-stage hMOL1, and hMOL3 developed via hMOL2 into end-stage hMOL5\textsuperscript{42} (Fig. 2b). Surprisingly, myelination-related genes were highly expressed in the two intermediate populations hMOL3 and hMOL4, and not in the maturation endpoint populations\textsuperscript{42} (Fig. 2b). This indicates that next to myelination, fully matured wmOLGs likely have other important functions not yet identified\textsuperscript{42,43} that may relate to myelin maintenance and/or function in synaptogenesis. Another possibility is that these two fully mature OLG populations may actively support neuronal functioning. Indeed, OLGs are suggested to provide trophic support to neurons\textsuperscript{44}, and OLGs that have formed myelin membranes actively transport glycolysis products from the blood stream to the myelinated axon via monocarboxylate transporters 1 and 2\textsuperscript{45}. In addition, expression of the monocarboxylic acid transporter MCT1 in OLGs is required for neuronal survival and function\textsuperscript{46}. Notably, in the healthy brain, hMOL6 are most abundant on the border between GM and WM\textsuperscript{44}. While in the study of Jäkel and co-workers\textsuperscript{47,48} solely WM tissue was studied, in another recent snRNAseq study GM, WM and leukocortical MS lesions were analyzed and compared to control\textsuperscript{49}. In this study one group of OPCs and one group of OLGs were identified in control tissue. As this paper however focused on differences between control and MS tissue, the authors did not elaborate on potential differences between control GM and WM\textsuperscript{44}. Hence, whether in humans also an enrichment for one of the hMOLs in GM or WM as in mice exists remains to be determined.

Thus, in contrast to OPCs, mature OLGs not only differ in their morphology but are also heterogeneous at the transcriptional level. Remarkably, all 6 MOL populations are present in the juvenile and young adult mice, while also 6 MOL populations are present in middle-aged human brain tissue, suggesting that they are formed during development. As a consequence, the two divergent maturation hMOL patterns may have a different myelinogenic potential, i.e., differences in composition, number and length of myelin segments. Although the myelinogenic potential of the mMOL and hMOL populations has not been addressed yet, the myelinogenic potential of OLGs in different brain regions in vivo has been described, which will be discussed next.
Figure 2. Schematic representation of oligodendroglial lineage cell subtype clusters in murine and human physiological and pathological conditions. (a) Single-nuclei RNA-sequencing identified oligodendroglial lineage cell subtype clusters in 10 different regions from the adult mouse central nervous system. A single cluster of oligodendrocyte progenitor cells (mOPC1) differentiates into a single cluster of differentiation committed OPCs (mCOP). This is followed by 2 stages of newly-formed oligodendrocytes (mNFOL1/mNFOL2) and 2 stages of myelin forming oligodendrocytes (mMFOL1/mMFOL2). Real diversification, as opposed to sequential maturation stages, occurs in the last stage and is apparent as 6 mature oligodendrocyte stages (mMOL1-6). Of these, mMOL1-4 expressed myelination and lipid biosynthesis genes, while mMOL5 and mMOL6 expressed synapse related genes. Upon induction of experimental autoimmune encephalomyelitis (EAE), an animal model for MS diversity in myelinogenic potential?

In vivo analysis of single cell shows that OLGs in a given region display a great diversity in the number of myelin segments, while the length of each myelin segment formed by an individual OLG also varies. Although OLGs in the cerebral cortex form a slightly higher mean number of myelin segments per OLG and a seemingly shorter myelin segment length compared to OLGs in the corpus callosum, the myelinogenic potential appears not to be region-specific. This indicates that the number and length of myelin segments is likely regulated by micro-environmental cues. Indeed, neuronal activity-mediated regulations of intracellular Ca\(^{2+}\) concentrations affect myelin sheath development. Other factors that may affect the number of axons myelinated and the length of the myelin segments are axonal caliber and OPC competition. For example, in the corpus callosum of the rat, OLGs myelinate more axons (9.6 versus 6.7 axons on average) and have shorter internodes (79.1 µm versus 106.1 µm) compared to WMOLGs in the cerebellum, likely because axons in the corpus callosum have a smaller diameter than those in the cerebellar WM. In addition, studies in rodents and cats have shown that larger axons provoke the production of longer, but fewer, internodes by OLGs. Moreover, the density of OPCs also regulates the myelinogenic potential. The abundance of OPCs has a negative correlation with the number of myelin segments, a process mediated via Nogo-A. In addition, OLs that myelinate nanofibers in vitro adapt myelination patterns to the nanofiber diameter; the myelin sheath length increases with nanofiber diameter. It is hypothesized that adapting myelination to axonal size is an evolved trait. Motor output, which is critical for fast reactions upon threats, requires higher conduction speed than less critical data movement between the cerebral cortices. Hence, the first is signaled over thicker, and the latter over thinner, axons. This
evolutionary advantage might also underlie (1) the differences in myelination-level of the adult CNS, i.e., the optic nerve consists of almost only myelinated axons\textsuperscript{178} and the cortex and corpus callosum contain both myelinated and unmyelinated axons\textsuperscript{179,180}, and (2) the timing and duration of myelination as suggested by neuroimaging and cell age studies\textsuperscript{181-183}. For example, in humans the volume of WM increases up to 19 years of age\textsuperscript{184}, while myelination of GM areas is not complete until the fourth decade of life. Surprisingly, a recent study showed that the number of OLGs in mice is almost twofold higher in the corpus callosum than in the almost completely myelinated optic nerve, while OLG survival in these regions is comparable\textsuperscript{185}. This is possibly due to a higher amount of myelination-stimulating signals from the higher number of naked receptive axons\textsuperscript{186}. Not only the number of naked axons differs between regions, also the direction of these axons; while in the axon bundles of WM tracts myelination is characterized by OLG processes that align with axons, the orientation of myelin segments in the GM is more omnidirectional as axons in the GM are not uniformly aligned. On the other hand, the source of OLGs influences their myelination pattern, i.e., cultured OLGs derived from the spinal cord generate longer sheaths compared to OLGs from the cortex\textsuperscript{187}, pointing also to intrinsic differences in OLGs from different regions. Also differences between myelination during development and in the adult CNS have been observed. More specifically, myelinating OLGs that have developed in the optic nerve during adulthood show more and shorter internodes\textsuperscript{188}. Possibly, newly-produced OLGs in the adult brain either replace dying OLGs or incorporate between the pre-existing myelin segments and in this way the total number of contributing OLGs increases\textsuperscript{189}. While it is likely that axonal signals that determine myelin segment length and thickness are lacking or less prominently present in the adult than in the developing CNS, it cannot be excluded that reported differences between neonatal and adult OPCs\textsuperscript{188} may contribute.

Whether myelin composition differs between different regions has not been thoroughly analyzed yet. It has been observed that human WM homogenates, i.e., that contain cells and myelin, are relatively enriched in lipid content (54.9% in WM versus 32.7% in GM), while human GM homogenates are more enriched in protein (55.3% in GM versus 39.0% in WM). Notably, fatty acids such as ethanolamine and serine glycerophosphatides, and lecithin are more abundant in GM than in WM homogenates, while cholesterol, sulfatide and cerebroside levels are higher in the

WM lipid fraction\textsuperscript{151,152}. Whether this reflects the lower myelin content in GM, or differences in myelin composition, and thus heterogeneity of GM and WM myelin per se, remains to be determined. In favor of the latter, myelin protein concentrations and myelin protein activity from distinct human brain regions differ more than the regional discrepancy in myelin content accounts for\textsuperscript{153}. Although the concentration of PLP, MBP and activity of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) is higher in WM homogenates than in their regional GM counterparts, the fold difference ranges between 3.3x for CNP (frontal GM versus WM) and 9.6x for MBP (frontal GM versus WM), which may point to a regional heterogeneity in myelin composition. This hypothesis is supported by differences in the lipid percentage ratio which differs between 1.3x for cholesterol and 3.7x for cerebroside (table 1).

Table 1. Regional concentrations or activity of myelin proteins and lipids (adapted from \textsuperscript{183}).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Frontal GM</th>
<th>Frontal WM</th>
<th>FD*</th>
<th>Temporal GM</th>
<th>Temporal WM</th>
<th>FD*</th>
<th>Corpus callosum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP (µg/mg protein)</td>
<td>95.8</td>
<td>488.8</td>
<td>51.1x</td>
<td>48.2</td>
<td>398.2</td>
<td>8.3</td>
<td>516.8</td>
</tr>
<tr>
<td>MBP (µg/mg protein)</td>
<td>22.8</td>
<td>218.0</td>
<td>9.6</td>
<td>23.5</td>
<td>155.5</td>
<td>6.6</td>
<td>178.2</td>
</tr>
<tr>
<td>CNP (U/mg protein)</td>
<td>4.7</td>
<td>15.4</td>
<td>3.3</td>
<td>3.5</td>
<td>16.2</td>
<td>4.6</td>
<td>15.5</td>
</tr>
<tr>
<td>Lipid (% of total dry weight)</td>
<td>GM*</td>
<td>WM*</td>
<td>FD*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>22.0</td>
<td>27.5</td>
<td>1.3x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebroside</td>
<td>5.4</td>
<td>19.8</td>
<td>3.7x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfatide</td>
<td>1.7</td>
<td>5.4</td>
<td>3.2x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = Fold difference (FD) in concentration or activity between regional GM and WM

Plasticity of myelin is also observed during aging. The abundance of MBP decreases in healthy human aging\textsuperscript{189,190} and even more in patients with Alzheimer's disease\textsuperscript{191}. In contrast, in aged rhesus monkeys, MBP levels remain unchanged, whereas CNP levels increase in aged rhesus monkeys\textsuperscript{192}. How this plasticity in composition affects the quality of myelin is yet unknown and whether this is different among species are interesting areas of future research.
Taken together, while OPCs are transcriptionally less diverse, mature OLGs intrinsically differ and constitute a heterogeneous group of locally established cells (Fig. 2). The diversity in OLGs may determine myelination efficiency in GM versus WM. Indeed, where wmOLG lineage cells produce myelinating wmOLGs in the WM, in the GM, the majority of gmOLG lineage cells remain in an immature Ng2-positive stage. Whether the variety in myelin phenotype may also be a product of intrinsic differences in the myelin-producing cell, i.e., the OLG, in conjunction with axonal cues that orchestrate differences in myelogenic potential remains to be investigated. Indeed, a cellular phenotype is a product of the interplay between intrinsic identity and extrinsic environmental cues. In addition to axonal cues, also regional cues of other cell types, such as regional diverse ASTRs, may affect the diversity of oligodendroglia during development, aging or upon response to demyelinating injury.

**Astrocyte diversity**

*Astrocytes subtypes in the grey and white matter of the adult brain*

Originally, ASTRs are divided into two groups based on their morphology and relates to region; fibrous ASTRs reside in WM and protoplasmic ASTRs are present in GM (Fig. 1). Protoplasmic gmASTRs are morphologically complex with a high number of fine processes that ensheathe synapses and usually have one or two processes in contact with the microvasculature. Fibrous wmASTRs are less complex, and have fewer branching processes, but long, thin processes, yielding a star-like appearance. This morphological difference is accompanied by differences in the abundance of the intermediate filament protein GFAP, which is more prominently present in wmASTRs than in gmASTRs. The distinct ASTR subtypes may relate to their distinct function in either area. Fibrous wmASTRs seem specialized in providing structural support for myelinated axons, as they have numerous overlapping processes combined with evenly spaced cell bodies. They are organized along WM tracts and longitudinally oriented in the plane of fiber bundles. Moreover, wmASTRs make contact with blood vessels and nodes of Ranvier. Consistent with their lack of synaptic contact, fibrous wmASTRs possess less fine bulbous processes than protoplasmic gmASTRs. Protoplasmic gmASTRs are eveny distributed throughout the cortex and bear their own micro-domain with hardly any overlap between neighboring cells. Even though the exact role of the micro-domain organization is not clear, its architecture suggests a prominent role in coordination of synaptic activity and blood flow, potentially independent of neuronal metabolic activity. Next to morphological differences between gmASTRs and wmASTRs, they may also differ functionally in for example glutamate handling. In the rodent brain, capillary density and branching is 3-5 times higher in the GM versus WM, which is accompanied by a lower BBB permeability in the GM versus WM. Additionally, each rodent protoplasmic gmASTR covers between ~20,000-120,000 synapses, whereas a human gmASTR can cover from ~270,000 to 2 million synapses, which may improve memory and learning.

The classification of ASTRs into protoplasmic and fibrous ASTRs may be a simplified representation of ASTR subtypes. After the early discovery of ASTRs in 1913, Cajal divided ASTRs into different subclasses with a staining method using gold chloride which stains both ASTRs and neurons, and classified them based on their morphology and contact with blood vessels. In 2006, an in depth morphological and biochemical analysis by Emsley and Macklis divided ASTRs into nine different classes based on morphology, GFAP, and Suxol expression. Adding to the complexity of ASTR form and functions, human and primate ASTRs are 2.6-fold larger in diameter and 15.6-fold larger in volume compared to rodent ASTRs. As this increase in size is valid for both fibrous and protoplasmic ASTRs, this may represent an evolutionary optimal increase relative to the increase in total brain size. Also, human ASTRs extent 10-fold more GFAP-positive primary processes than their rodent counterparts. In addition, primates and humans have more subtypes of ASTRs compared to other mammals. Primates harbor two extra types of glia in the cortex; interlaminar ASTRs and varicose projection ASTRs. Up to this point, varicose projection ASTRs have only been observed in humans and chimpanzees. On the other hand, interlaminar ASTRs are specific for primates and of these most abundantly present in humans. The function of these two ASTR subtypes is unknown, but it is suggested that they provide a network for the long-distance coordination of intracortical communication thresholds and play a role in coordinating blood flow. Surprisingly, although many different morphological and functional subtypes of ASTRs are described, in murine scRNAseq and human snRNAseq studies of WM only two to three groups...
of transcriptionally different ASTRs are defined\textsuperscript{20,21,22}, based on specific marker expression like Gfap and Mfge8 in mice\textsuperscript{21}, Gpc5 for human gmASTRs and Cd44 for human wmASTRs\textsuperscript{20}. Using reporter mice and a FACS sorting panel of 81 cell surface antigens, John Lin and coworkers describe five different ASTR populations based on ASTRs isolated from cortex, cerebellum, brainstem, olfactory bulb, thalamus and spinal cord\textsuperscript{20}. These five groups display, next to surface antigen expression, also functional differences. Gene expression profiling of these five groups reveals that although the five ASTR populations are functionally and morphologically different, three of the five ASTR populations appear transcriptionally similar, hence indicating ASTR plasticity of a comparable transcriptional population. Combined with the other 2 remaining groups, in this study 3 intrinsic, transcriptionally heterogeneous populations are described. Of these, one population was more abundant in the cortex. Hence, diversity of form and function is not solely based on intrinsic heterogeneity, but may come from ASTR plasticity\textsuperscript{20}. Finally, not only ASTR phenotypes varies greatly, but also ASTR density varies between different brain regions. In mice, the density of ASTRs is highest in the subventricular zone (2500 cells/mm\textsuperscript{2}) and ASTRs in the corpus callosum are more dense than ASTRs within the cortex (~80 versus ~10 cells/mm\textsuperscript{2}, respectively)\textsuperscript{20}, indicating that different local functional demands require different numbers of ASTRs.

**Astrocyte coupling in the grey and white matter**

ASTRs are coupled to each other by homotypic gap junction coupling via connexin 43 (Cx43) which is expressed on both gmASTRs and wmASTRs, and to a lower extent via Cx30 which is only expressed by gmASTRs\textsuperscript{22}. Gap junctions with Cx30 and Cx43 on ASTRs, respectively. Although both gmOLGs and wmOLGs express Cx32 and Cx47, their expression is higher in wmOLGs\textsuperscript{24}. The coupling of ASTRs/ASTRs as well as the coupling ASTRs/OLGs increases during development\textsuperscript{20}. ASTR Cx43 coupling to OLGs may play a role in myelin maintenance and suggested to play a role in redistribution of K\textsuperscript{+} after neuronal activity. Indeed, OLG gap junction ablation\textsuperscript{22,23} and/or the deletion of potassium channel Kir4.1 on OLGs\textsuperscript{23,24} cause vacuolation of myelin. Gap junctions between ASTRs and OLGs are crucial for developmental myelination and survival of OLGs\textsuperscript{25,26,27} especially in the WM\textsuperscript{28}. In mice, a double knock-out of astrocytic Cx43 and Cx30 results in widespread pathology of WM tracts during development which persists with aging, and includes vaculated OLGs and intramyelinic oedema\textsuperscript{29}. Contrastingly, GM pathology was only observed in part of the hippocampus, which was restricted to edematous ASTRs. Thus, in GM, gap junctions between ASTRs and OLGs seem less important for OLG survival and myelin maintenance\textsuperscript{29}, which may be reflected in the lower expression of Cx32 and Cx47 in gmOLGs\textsuperscript{28}.

Taken together, based on gene expression, morphology and function, a variety of ASTR phenotypes can be discerned with region as an important determinant. ASTRs are one of the first responders to CNS injury, and the ASTR subtypes may differently respond and/or have different functions upon e.g., demyelination in GM and WM. In turn, OPCs and mature OLGs from different regions may act differently in response to changes in their microenvironment, including to ASTR-derived changes, and their ability to remyelinate, which will be reviewed next.

**Diversity of regional macroglia upon CNS demyelination and remyelination in rodent models**

**Remyelination in the grey and white matter**

Regional differences in macroglia affect cells’ responses towards injury, and may therefore play an important role in the extent of disease pathology and recovery. For example, gmOPCs, but not wmOPCs, take up mercury, which may involve ASTR-mediated trafficking of mercury via gap junctions\textsuperscript{30}. A valuable model to study regional diversity in macrogliae responses upon demyelinating CNS is the cuprizone model\textsuperscript{22}. In this model mice are fed with cuprizone, leading to reversible global demyelination in GM and WM, of which the cortex and corpus callosum are most studied\textsuperscript{22}. Asspontaneous and robust remyelination is observed following withdrawal of the toxin, these models have provided insight in the process of remyelination. In
Role of macroglial diversity in remyelination

Chapter 1

1

rodent remyelination, adjacent OPCs are transcriptionally activated and recruited to the area of demyelination, where they differentiate into myelinating OLGs, a process orchestrated by signaling from local microglia and ASTRs. When administered to young adult mice, cuprizone induces a different de- and remyelination phenotype in GM and WM. More specifically, there is a delay peak of initial and complete demyelination in the cortex compared to the corpus callosum. Several studies report that remyelination is more efficient in the corpus callosum than in the cortex upon cuprizone intoxication. However, a limitation of the cuprizone model is that after initial demyelination, myelin debris clearance parallels an early process of remyelination, and that mature OLGs appear regardless of whether the cuprizone diet is maintained or not. Therefore, as demyelination is delayed in the cortex, likely also the re-expression of myelin proteins as well as remyelination is delayed in the cortex, frustrating the comparison of differences in regional remyelination upon cuprizone feeding alone. Interestingly, upon co-administration of cuprizone combined with rapamycin the remyelination process is not occurring until treatment cessation. Under these conditions, i.e., when the remyelination process starts at the same time in GM and WM regions, remyelination proceeds faster in the cortex than in the corpus callosum. Hence, the timing of demyelination and efficiency of remyelination are distinct in GM and WM areas. Notably, the differences in the time-course of de- and remyelination is also a heterogeneous process within GM itself, i.e., in cingulate cortex and hippocampus the timing and speed of remyelination differs upon cuprizone-induced demyelination. Whether regional diversity of local macroglia responses may contribute to more efficient remyelination in GM than in WM is discussed next.

Local OPCs and remyelination

Differences in regional remyelination capacity in experimental rodent models may be explained by the intrinsic differences of regional OPCs. For instance, dorsally derived OPCs have a higher remyelination capacity than ventrally derived OPCs in vivo and an enhanced capacity to migrate and differentiate in vitro. Also, the expression of G-protein coupled receptor 17 (Gpr17) is induced on wmOPCs, but not on gmOPCs, upon cuprizone induced demyelination. Gpr17 is expressed on maturing wmOLG lineage cells, where it is involved in the initiation of differentiation. The timely downregulation of Gpr17 is required for terminal wmOLG maturation and myelination. Hence, Gpr17 may play a central role in orchestrating repair processes in the WM, but not the GM, including remyelination. Importantly, rodent adult OPCs respond to demyelinating injury by reverting to a simpler morphology and a more immature state at the transcriptional level before differentiating and, ultimately, remyelinating denuded axons. In addition, activated adult OPCs show increased migration and accelerated differentiation compared to resting adult OPCs. Moreover, activated adult OPCs directly regulate their recruitment to demyelinated areas by increasing their expression of IL1β and CCL2. Notably, regional differences were not taken into account and IL1β and CCL2 expression is only verified in wmOLG.
Astrocyte diversity and remyelination

In addition to diversity of OPCs from different regions, ASTR responses towards demyelination may also vary between ASTRs from different regions. Upon OLG loss and demyelination, ASTRs become reactive, which in the cuprizone model involves ASTR reactivity in the corpus (CNTF, BDNF) or are preferentially expressed during demyelination in the cortex (FGF2, HGF). Notably, CNTF and BDNF accelerate OPC maturation and FGF2 and HGF both enhance OPC proliferation and migration and prevent their differentiation. Thus, remyelination efficiency depends on intrinsic differences between gmOPCs and wmOPCs as well as on the availability of signaling factors, such as growth factors, to respond to. While differences in gmOPC and wmOPC responses towards demyelination-relevant injury signals are evident, differences in response of the distinct mature myelinating OLG populations towards CNS injury have not been reported yet. As ASTRs are the cellular source of CNTF, BDNF, HGF, and FGF2, ASTR diversity may contribute to the differences in remyelination efficiency in GM and WM.

Astrocyte diversity and remyelination

In addition to diversity of OPCs from different regions, ASTR responses towards demyelination may also vary between ASTRs from different regions. Upon OLG loss and demyelination, ASTRs become reactive, which in the cuprizone model involves ASTR proliferation, upregulation of reactive ASTR markers, such as GFAP and vimentin, and the elaboration of a dense network of processes. In experimental demyelination models, ASTR reactivity is more prominent in the corpus callosum than in the cortex, though ASTR reactivity has been suggested to start earlier in the cortex. ASTR reactivity is regulated by pro-inflammatory cytokines, Toll-like receptor (TLR)-mediated signaling events, and myelin debris. As the BBB remains intact in the cuprizone model, most inflammatory mediators that induce ASTR reactivity are provided by microglia. Indeed, in the cuprizone model, microgliosis precedes loss of OLGs and is in the corpus callosum already apparent when myelin still appears normal. In contrast, in the cortex microgliosis activation is less prominent and delayed. Hence, early microgliosis activation precedes ASTR reactivity in the corpus callosum, while ASTR reactivity is already evident when microgliosis activation peaks, indicating that ASTR reactivity in GM and WM is heterogeneous as a consequence of differential inducing signal factors. Of note, both in the corpus callosum and cortex, transcripts of the chemokine CCL2 are transiently enhanced early upon cuprizone administration, while mRNA levels of CCL3 continuously increase. However, when CCL2 and CCL3 are both absent, ASTR reactivity and demyelination in the cortex, but not in the corpus callosum, are both reduced, which is in line with the assumption that ASTR reactivity in GM and WM is different and distinctly modulate de- and remyelination.

Indeed, ASTR reactivity is heterogeneous, and depends on the type of injury and the inducing mediator(s). Reactive ASTRs have been classified as anti-inflammatory A2-ASTRs, induced by myelin debris and/or TLR3 agonists, and characterized by SsooA2, pro-inflammatory A1-ASTRs induced by microglia-derived IL-1α, TNF and Cq and characterized by C3. Mild activation of ASTRs may induce a pro-reparative A2-ASTRs, while reactive A1-ASTRs inhibit OPC proliferation, migration and differentiation and secrete toxic factors for OLGs. Transgenic over-expression of GFAP expression alters the chemokine secretory profile by ASTRs and protects against cuprizone-induced demyelination in the corpus callosum while the authors did not look into the GM. ASTR reactivity that is correlated with an upregulation of GFAP may serve a protective function. Another feature of reactive ASTRs is increased deposition of extracellular matrix (ECM) proteins. Upon toxin-induced demyelination, ASTRs transiently deposit several ECM proteins, including CSPGs and fibronectin, which add to resolve injury and recovery. The composition of the ECM affects OPC behavior; fibronectin increases OPC proliferation and migration and inhibits OPC differentiation, while CSPGs inhibit OPC proliferation, migration and differentiation. Differentiation of neural stem cells into OPCs and finally into mature myelinating OLGs, is in addition to composition, also dependent on the stiffness of the ECM. A rigid matrix promotes OPC proliferation and early differentiation, while a soft matrix favors OLG maturation and myelination. Regional differences in stiffness have been observed. Thus, WM is more stiff compared to GM which is, among others, due to a higher abundance of myelin. Notably, in the cuprizone model, a decreased stiffness in the corpus...
callosum is observed upon acute demyelination, while in chronically cuprizone-induced demyelinated lesions that fail to remyelinate, an increase in ECM deposition and tissue stiffness is measured\(^{45}\). Therefore, enhanced deposition of ECM proteins in the corpus callosum may contribute to recruitment and early differentiation of OPCs, but removal of these proteins is required for OLG maturation and myelination. ECM proteins are degraded, among others, by metalloproteinases (MMPs), which are mostly expressed by microglia and ASTRs\(^{37}\). In the cuprizone model, ASTRs in the corpus callosum express both MMP3 and MMP12 during remyelination, while less or no expression was detected in ASTRs in the cortex\(^{45}\), indicating that ECM remodeling by these MMPs is more relevant in WM than in GM during remyelination. Hence, it is tempting to suggest that a regional difference in inducing stimuli and in ECM remodeling of gmASTR and wmASTR during reactive gliosis\(^{17,26}\) may add to regional difference in remyelination efficiency in the cortex and corpus callosum in the cuprizone model.

Taken together, in experimental models, the difference in regional remyelination efficiency may be explained by OPC heterogeneity and plasticity, while the role of regional ASTRs relate more to context of injury and local inducing stimuli. A potential role of pre-existing heterogeneity of gmASTRs and wmASTRs remains to be determined. Our unpublished observations showed that both neonatal and adult wmASTRs are less supportive for in vitro myelination than neonatal and adult gmASTRs. Whether macroglia diversity and their interactions also play a role in remyelination efficiency in GM and WM MS lesions, will be described next (summarized in Fig. 3).

Macrogial diversity; implications for MS

**Remyelination in MS lesions**

MS is a chronic progressive disease of the CNS characterized by the formation of demyelinated lesions that, upon failure of remyelination, ultimately lead to neurodegeneration and increasing state of neurological disability. In MS, substantial remyelination is reported to occur at any given age, even well into the 8th decade of life\(^{7,8,37,168}\). However, remyelination efficiency is variable; lesions are mostly efficiently repaired in the early stages of MS, but often limited upon aging and when the disease progresses\(^{4,6–8}\). Possible explanations for the decrease in remyelination efficiency include failure of OPCs to migrate to the lesion, failure of OPC differentiation into myelinating OLGs, and/or failure of OLGs to effectively remyelinate axons\(^{5,9,10,26,18,27}\). In 70% of WM MS lesions OPCs are present but failed to myelinate the denuded axons\(^{98}\). This indicates that remyelination is not limited by an insufficient amount of OPCs, but rather a failure in maturation of OLGs\(^{48}\). Recent snRNAseq studies confirmed that OPCs in MS lesions are indeed relatively quiescent on a transcriptional level\(^{140}\). Experimental toxin-induced demyelination models show that the speed of remyelination, as other regenerative processes, decreases with age\(^{15,97}\). OPC characteristics affected by aging may contribute to impaired OPC differentiation. For example, CREB signaling in wmOPCs is impaired upon aging as observed in a mouse model of prolonged WM cerebral hyperperfusion\(^{171}\). A recent study shows that aged rat OPCs obtained from whole brain acquire classical hallmarks of cell aging, including increased DNA damage, decreased metabolic function, and become irresponsive to pharmacological-applied differentiation signals, such as miconazole and benzatropine\(^{172}\). In MS, patients with a more aggressive form of MS (shorter disease duration) show a smaller proportion of remyelinated lesions\(^{6}\). In addition, less remyelinated lesions are detected in progressive MS than in RRMS, and the proportion of remyelination is also lower in patients with cortical GM lesions\(^{6}\). The observation that myelination in the adult CNS is accompanied by more and shorter internodes, and that the produced myelin is thinner, is also observed in remyelinated MS lesions\(^{373}\). This might imply that this is in fact a feature of adult myelination, rather than an impaired myelin phenotype in remyelination\(^{6}\). Remarkably, carbon dating studies on WM brain tissue showed that newly-formed OLGs, i.e., generated from adult OPCs, are only detected in a small subgroup of patients that had an aggressive form of MS\(^{8}\). Intriguingly, in WM-derived shadow plaques, remyelinated areas\(^{78}\), newly-formed OLGs are absent, indicating that remyelination is not performed by adult OPCs, but by mature pre-existing OLGs generated during development from neonatal OPCs\(^{8}\). This is in line with a study in disease models of cats and non-human primates that show that at the electron microscopic level mature OLGs are connected to myelin sheaths of different thickness, hence are connected to myelin sheaths generated during both development and remyelination\(^{370}\). Whether the contribution of ‘old’ pre-existing mature OLGs to remyelination is specific to WM, or whether...
this is an adaptation, i.e., a ‘gain-of-function’ of mature OLGs as of the quiescence of OPCs in WM lesions remains to be determined.

**Differences in remyelination of grey and white matter MS lesions**

Historically, MS was considered mainly a disease of the WM, but now it has been well recognized that also GM pathology is prominent in MS. While GM lesions are categorized on basis of their location, the distinct WM lesions are classified by demyelination and inflammatory activity\(^6-12\). GM MS lesions are characterized by the loss of OLGs in the presence of a seemingly intact BBB, axonal loss, mild astrogliosis and reduced lymphocyte and macrophage infiltration\(^13-17\). By contrast, WM MS lesions are characterized by a variable infiltration of lymphocytes and macrophages, glial scar formation (Fig. 3) and microglia activity\(^18,19\). Hence, as in toxin-induced demyelination models, GM and WM demyelination and remyelination is likely different due to differences in the abundance and timing of inducing signal factors. To study differences in regional remyelination, leukocortical lesions, i.e., lesions that span both GM and WM areas, are therefore of special interest, as these lesions have a similar pathological background and age when comparing the GM to the WM area. Interestingly, similar to what is observed in the cuprizone model\(^6,20\), remyelination in the GM cortical portion of leukocortical MS lesions has a higher remyelination capacity than the WM non-cortical part\(^21\) (Fig. 3). In addition, the OLG density is 6.8 fold higher in the GM part than the WM part\(^22\). Also, the number of CSPG4-positive cells, which are mainly OPCs, is reduced in WM MS lesions compared to normal appearing WM (NAWM)\(^23\), while the number of OPCs is comparable between normal appearing GM (NAGM) and GM MS lesions, and even higher than in control GM\(^24\). Furthermore, astrogliosis and the expression of OLG differentiation inhibiting ECM components is higher in the WM part compared to the GM part of leukocortical MS lesions, while microglia are also more reactive in the WM part, possibly contributing to the differential effect on remyelination\(^25\). These findings are not restricted to leukocortical lesions, i.e., also in non-leukocortical GM MS lesions remyelination is more pronounced than in WM MS lesions\(^26,27\). Interestingly, transcriptome analysis of CNS cell types on postmortem tissue of different brain regions of MS patients and healthy subjects, shows more disease-related changes in the corpus callosum than in the cortex, most prominently in myelinating OLGs, but also in ASTRs\(^28\), hinting at diversity of OLG responses. Combined, these studies indicate that in remyelination, OLGs, but not OPCs, show the most intrinsic regional heterogeneity, ASTRs reactivity is regional distinct and depend on the presence of injury signals which likely differ in GM and WM, including the amount of myelin, and that there are less reactive microglia. What molecular differences underlie OLG diversity is discussed next.

**Oligodendroglia diversity in WM MS lesion pathology**

Two independent snRNAseq studies on post-mortem WM brain tissue found significant differences in transcriptome of mature OLGs in WM MS lesions compared to WM tissue of healthy subjects, while OPCs are transcriptionally quiescent\(^29,30\). More specifically, of the six identified mature hMOL populations in control human brain tissue, the fully mature hMOL1, which does not express high levels of myelination related genes, and the more immature hMOL6 population are less abundant in WM MS lesions, while imOLG, hMOL2, hMOL3 and hMOL5 are enriched in WM MS lesions (Fig. 2b). In addition, increased transcript levels of myelin genes are observed in mature wMOs in MS\(^31\), hinting to the involvement of mature OLGs in remyelination. Thus, in WM MS lesions, some hMOLs populations are skewed to the transcriptionally different fully mature hMOL5 population, or that other populations are depleted as adult OPCs lack the capability or receive inhibitory signals to form new hMOLs\(^32\), which may represent the reduced abundance of the pre-myelinating hMOL6\(^3,33,34\). Spatial analysis of MS lesions showed that genes related to OPC differentiation are reduced in WM lesions borders and that genes belonging to stress pathways and related to iron accumulation are increased\(^35\). Although not studied\(^36\), nor extensively discussed yet\(^37\), given that OPCs are more abundant in GM MS lesions\(^38,39\) and the selective appearance of stressed OLGs in WM lesions borders\(^40\), it is tempting to suggest that remyelination in GM may proceed via newly-formed OLGs and remyelination in WM MS lesions via pre-existing OLGs.

In both snRNAseq studies on MS brain tissue, an immunocompetent phenotype can be observed in OLG lineage cells from all maturation stages, and include increased transcript and protein levels of major histocompatibility complex class I (MHC I)\(^41,42\) and MHC II\(^43,44\). An immunocompetent phenotype of both OPCs and OLGs is also present in experimental autoimmune encephalomyelitis (EAE), an animal model that resembles autoimmune inflammatory aspects of MS\(^45\). scRNAseq of spinal
cords (sc)-derived oligodendroglia at the peak of EAE identified 8 mature sc-OLG clusters, of which five are EAE-associated, including a sc-mMOL cluster that mainly comprises newly-formed OLGs (Fig. 2a). Further analysis uncovered an EAE-specific gene module that is restricted to the newly-formed OLGs population and another module that comprises genes associated with the IFN response pathway and MHC-I and MHC-II genes (Fig. 2a). Strikingly, in contrast to control spinal cord tissue where only one sc-mOPC cluster is present, three additional sc-mOPC clusters are observed in EAE tissue. Of the three EAE-specific sc-mOPC clusters, one is a cycling sc-mOPC population, while transcription levels of myelination related genes are increased in the other two sc-mOPC populations, indicating that these sc-mOPCs were transferred from a quiescent state to actively differentiating (Fig 3a). Notably, in WM MS lesions transcriptionally different OPC populations are lacking, indicating that in contrast to EAE, in MS OPCs are likely not activated and triggered to differentiate. Alternatively, given that most MS lesions are likely relatively old, and may have had their initial demyelinating event months to years ago, it cannot be excluded that in newly emerged MS lesions OPCs are more diverse. Surprisingly, EAE-associated mOPCs also express MHC-II genes of which induction is mediated by IFNγ in vitro. IFNγ also induces MHC-I expression in gmOPCs, and these MHC-I expressing OPCs present antigens to cytotoxic T cells. Given that wmOPCs are more susceptible to IFNγ than gmOPCs in vitro, it would be interesting to investigate if wmOPCs also showed MHC-reactivity upon exposure to IFNγ. Of relevance, EAE-associated mOPCs exhibit phagocytic activity, take up myelin debris and likely present myelin-specific antigens. Of note, bulk-RNAseq did not reveal an upregulation of MHC-I or MHC-II genes in adult OPCs upon cuprizone-induced demyelination. Hence, it would be interesting to investigate with snRNAseq whether upon cuprizone-induced demyelination distinct OPC clusters can be identified. Of note, bulk-RNAseq of OPCs reveals upregulation of genes associated with the innate immune system, such as IL1β and CCL2, upon cuprizone-induced demyelination. In conclusion, the upregulation of immunomodulatory genes in OLG lineage cells suggests that these cells may have a more direct role in MS disease origin and progression, and contribute to OLG heterogeneity. Alternatively, the upregulation of immunomodulatory genes in OLG lineage cells may represent a natural transient response towards demyelination, but persists in MS. In addition, in EAE, but not MS, OPCs are transcriptional active, indicating that transcriptional activation of OPCs in MS lesions is likely impaired.

Astroglial scar formation WM MS lesions

ASTRs change their phenotype in demyelinated MS lesions as well, while astrogliosis may vary between GM and WM MS lesions. Indeed, phenotype clustering of ASTRs and myeloid cells, i.e., using mass cytometry and thirteen glia-related markers, revealed five different groups of ASTRs in MS lesions. Two of these were present in the center of GM and WM lesions, one on the inner GM and WM rim and one on the WM outer rim, and the final subgroup of ASTRs was present in NAWM. As the ASTR phenotypes locate to different zones of MS lesions, it is suggested that these phenotypes form functional diverse groups. As also shown in the snRNAseq studies that identified only two to three ASTR subclusters, the five different ASTR populations may be a representation of functional plasticity of the same ASTR subtype in MS, rather than a representation of intrinsic ASTR heterogeneity. The two ASTR clusters identified by snRNAseq represent protoplastic gmASTR, in MS lesions characterized by SCC1A2, and fibrous/reactive wmASTR that express more GFAP, CRYAB and MT3 in MS tissue. This indicates that astrogliosis is more apparent in WM MS lesions than in GM MS lesions. Indeed, the small heat shock protein CRYAB, also named HSPB5, supports the reactive ASTR response that contributes to demyelination in the cerebellum of the cuprizone model and is upregulated in active and chronic WM MS lesions, but not in GM lesions, in both the brain and the spinal cord. Together with a dense network of ECM proteins, hypertrophic ASTRs form a so-called astroglial scar around inflammatory WM lesions, but not GM lesions. This astroglial scar consist of new, proliferative ASTRs, which no longer occupy discrete domains and instead have overlapping processes that form a barrier against inflammation. The astroglial scar is usually considered as detrimental for remyelination. Interestingly, even though reactive ASTRs do emerge in the cuprizone model even beyond the demyelination period, the dense network of ASTR processes do no progress to form a barrier along the lesion even upon prolonged cuprizone exposure. Possibly, as a consequence of a distinct inflammatory profile, and local expressed inducing stimuli in GM and WM MS lesions, ASTR reactivity is increased in the WM part, as particularly evident in leukocortical lesions.
The astroglial scar in WM lesions mainly consists of interwoven astrocytic processes\(^\text{37}\). The processes of the ASTRs in the glial scar are highly filamentous, expressing high levels of GFAP, vimentin and nestin\(^\text{38}\). It appears that the function of a glial scar is to prevent spreading of inflammation to adjacent tissue, thus limiting further tissue damage (reviewed in \(^\text{39}\)). At the edges of active and expanding WM MS\(^\text{40}\), CSPGs are produced by ASTRs under control of regulator TRPM7\(^\text{41}\). The reduced remyelination capacity in the WM has been correlated with the accumulation of the CSPG versican, which is expressed by the wmASTRs, but not by gmASTRs in leukocortical lesions\(^\text{37}\).

A high number of cells positive for CSPG4, is found at the edge of the glial scar in chronic active lesions. Although often used as a marker for OPCs, CSPG4 expressing cells can also become ASTRs in vivo\(^\text{42}\), and in rodents also microglia initiate NG2 expression upon aging\(^\text{43}\). Other ECM proteins that impair OLG production and remyelination\(^\text{37,46,296–298}\) include hyaluronan and fibronectin. Interestingly, hyaluronan and its receptor CD44 are significantly increased in the WM, but not in the GM portion of leukocortical lesions\(^\text{37}\). Fibronectin is transiently expressed during toxin-induced demyelination models and aids OPC recruitment\(^\text{37,46,192,193,300}\) and newly-formed OLGs mature upon fibronectin degradation. However, while not detect in toxin-induced demyelination, ASTRs form the remyelination-imparing fibronectin aggregates in WM MS lesions. Aggregate formation is likely induced by insufficient fibronectin degradation\(^\text{39}\) combined with chronic inflammation\(^\text{46,39,304}\). Fibronectin aggregates persist in WM MS lesions, impairing OPC maturation and thereby contributing to remyelination failure\(^\text{39}\). Although GM MS lesions have not been studied in the context of fibronectin aggregates yet, fibronectin is not present in GM marmoset EAE lesions\(^\text{18}\). Also, in vitro more fibronectin aggregates are formed by wmASTRs than by gmASTRs, which may reflect intrinsic differences in alternative splicing of fibronectin between gmASTRs and wmASTRs\(^\text{39}\). It would be interesting to investigate whether the reactive astroglosis and the formation of a glial scar in WM MS lesions may account for decreased presence and/or differentiation to the fully mature hMOL1 as identified by the snRNAseq study\(^\text{12}\), and thereby contributing to the difference in remyelination efficiency in GM and WM MS lesions. Supporting this hypothesis, while in acute demyelinated lesions of both MS and experimental toxin-induced models tissue stiffness is transiently decreased \(^\text{37}\), thus forming an environment which supports active myelination\(^\text{47}\), chronic demyelinated MS lesions are stiffer than control tissue\(^\text{37}\) (Fig. 3), thus providing a myelination-inhibiting environment\(^\text{47}\). Regional differences in stiffness dynamics have also been reported in the chronic EAE model for inflammation mediated demyelination. Contrasting to chronic cuprizone and MS lesions, in EAE WM, an initial increase in stiffness is observed at EAE onset and peak phase of demyelination, which is followed by a decrease in stiffness in the chronic phase, while GM stiffness remains unaffected\(^\text{41}\).

### Astrocyte Signaling in Grey and White Matter Multiple Sclerosis Lesions

In experimental rodent models of successful remyelination, OPCs have to be recruited to the demyelinated areas. Semaphorins are extracellular signaling guidance proteins that play an important role in OPC recruitment. For example, semaphorin 3F (Sema3F) acts as a chemo-attractant and Sema3A as a chemo-repellent for OPCs\(^\text{39}\). In active WM MS lesions, Sema3A and Sema3F expression is increased in both reactive ASTRs and microglia\(^\text{39}\). Sema3F expression was abundant in highly inflamed WM lesions, whereas Sema3A expression was predominant in less inflamed lesions\(^\text{37}\). Both semaphorins are also detected in GM MS lesions, but here their expression is strictly neuronal\(^\text{37}\), indicating that semaphorins are differentially expressed by gmASTRs and wmASTRs. Upon toxin-induced demyelination in the spinal cord, lentivirally-mediated overexpression of Sema3A in ASTRs inhibits OPC recruitment to demyelinated areas and thereby prevents remyelination\(^\text{46}\). OPCs are present in 70% of WM MS lesions albeit in lower numbers than surrounding NAWM\(^\text{46,39,304}\). The lower abundance of OPCs may be below threshold for successful remyelination. Sema3A also inhibits OLG differentiation and when Sema3A is injected into focal toxin-induced demyelinated lesions OPC differentiation is halted at the pre-myelinating stage and these pre-myelinating OLGs contact axons, but fail to make myelin sheaths. Thus, Sema3A in MS lesions may in addition to diminishing OPC recruitment, also halt differentiation of OPCs in a pre-myelinating stage\(^\text{46}\). Of note, in the WM part of leukocortical lesion, pre-myelinating OLGs with multiple processes that are associated with demyelinated axons are present but fail to myelinate the denuded axons\(^\text{37}\). As gmASTRs do not express Sema3A, and as the expression of neuronal Sema3A may be differentially regulated and do not play a major role in OPC recruitment, the differential expression of semaphorins in gmASTRs and wmASTRs may contribute to increased remyelination efficiency in GM lesions. This is consistent with the abundant presence of OPCs in GM MS lesions\(^\text{46,37}\).
Also, regional differences in direct cell-cell contact via gap junctions between ASTRs and OLGs in MS lesions may contribute to differences in remyelination capacity. For example, the expression of ASTR connexins is altered in MS. In both GM and WM MS lesions, Cx43 is upregulated on ASTRs and also slightly increased in NAGM and NAWM\(^{30,39}\) (Fig. 3). Contrastingly, in EAE, a loss of Cx43 expression in ASTRs is observed in the spinal cord\(^{30}\). Remarkably, increased expression of Cx43 in GM MS lesions correlated with enhanced microglial activation\(^{30}\). In addition to Cx43, also the GM-specific Cx30 is upregulated in ASTRs in GM MS lesions, but not WM MS lesions\(^{30}\) (Fig. 3). In contrast, the expression of OLG Cx32 and OLG Cx47 is reduced in GM and WM MS lesions, with extension of the loss of Cx32 into NAGM and NAWM\(^{30}\). Cx47 expression is however increased on a number of OPCs in NAWM, although these OPCs have limited coupling to ASTRs\(^{30}\). Possibly this limited ASTR/OPC coupling is related to the failure of OPCs to differentiate into pre-myelinating OLGs\(^{30,39}\). Similar as in control GM, where gap junctions between ASTRs and OLGs seem less important for OLG and myelin maintenance than in WM\(^{30}\), remyelination in GM lesions may be less affected by the loss of connexin expression. Upon cuprizone induced-demyelination in the corpus callosum, Cx47 is initially upregulated in OLG processes, and in parallel a transient and de novo expression of Cx47 is observed in ASTRs\(^{30}\). Upon remyelination, Cx47 expression is switched back from ASTRs to the newly-formed OLGs\(^{30}\). Interestingly, ASTR signaling via Cx47 coupling to OPCs increases the expression of sphingosine-1-phosphate receptor 3 (S1PR3), which is activated by the lipid signaling molecule sphingosine-1-phosphate (S1P), and thereby promotes OPC proliferation\(^{30}\). Thus, the increased expression of Cx47 on OPCs in NAWM may aid proliferation of OPCs, which is in line with the observed increase of OPC numbers in NAWM\(^{30}\). On the other hand, ASTRs express S1PR1, also a receptor for S1P. In acute GM lesions, S1PR1 expression is decreased on ASTRs, while it remains present on ASTRs in WM lesions. Importantly, in GM lesions, S1PR1 reappears on ASTR when inflammation diminishes\(^{30}\). Moreover, the activation of S1PR1 on ASTRs promotes inflammation\(^{30}\), indicating that expression of S1PR1 plays a role in inflammation of WM, but not GM, MS lesions. Of note, treatment with fingolimod (FTY720), an inhibitor of S1PR, ameliorates EAE among others by inhibition of inflammatory activation of ASTRs and recovery of BBB function\(^{30,39}\). Hence, ASTR/OPC and ASTR/OLG interactions are distinct in GM and WM, and may contribute to regional differences in remyelination efficiency and are of relevance for the therapeutic effects.

**Concluding remarks and perspectives**

After initially being described well over a century ago, macroglial diversity has gained interest and momentum in the last few years which is relevant for understanding human pathology, including MS, where regional differences in remyelination efficiency are evident. Transcriptional profiling reveals that OLGs lineage cells become more heterogeneous upon maturation and may specialize in different location-dependent functions, i.e., myelination or synapse modulation\(^{30,39,40}\). While OPCs appear transcriptionally relatively homogeneous\(^{30,39,40}\), clear functional differences between gmOPCs and wmOPCs are reported, both in vitro and in vivo\(^{30,39,40}\). Whether and how regional diversity and interplay between macroglia from the GM and WM contribute to observed differences in remyelination efficiency, and MS pathology, is currently difficult to dissect. Demyelination of GM and WM areas induce different types of injury responses, with distinct local induced factors\(^{30,39,40}\), and as a consequence induces differences in ASTR reactivity. The roles of microglia and infiltrating immune cells have not been thoroughly discussed in this review, but are likely to be important players in inducing different type of injury response in either region\(^{30,39,40}\), in addition to the lower levels of myelin (debris)\(^{31}\) and the putative differences in myelin composition\(^{30,39}\) (Table 1) in GM compared to WM. Hence, more research both in vitro and in vivo are required to determine whether gmASTRs and wmASTRs respond differently to similar micro-environmental signals. For example, to exclude that the remyelination process is mainly dictated by the resident microenvironmental signals, homo- and heterotopic transplantation of either ASTRs in demyelinated GM or WM matter areas may be considered. Such transplantation studies have been performed...
for gmOPCs and wmOPCs in healthy adult brains, clearly hinting at intrinsic regional differences of OPCs in their maturation capacity\textsuperscript{119}. Transplantations of OPCs derived from different brain regions into demyelinated areas have not been reported yet, but given that gmOPCs are less mature and less susceptible for inflammatory cytokines, they may be better equipped for remyelination\textsuperscript{86,121}. Co-cultures of primary OPCs and ASTRs will also be valuable in understanding the role of macroglia diversity on the interplay between OPCs and ASTRs and its effect on myelination. Importantly, many \textit{in vitro} studies use GM-derived macroglia, while experimental models often focus on WM regions. This ignorance of macroglial diversity may lead to conflicting results obtained by \textit{in vitro} and \textit{in vivo} studies. In contrast to experimental models where remyelination is performed by newly-formed OLGs, remyelination in MS is performed by old, mature OLGs\textsuperscript{14}. Moreover, two transcriptionally distinct groups of OLGs are largely reduced in the MS brain\textsuperscript{12}, but the exact function of these two groups remains unclear. On the other hand, there is a gain of an immuno-OLG lineage phenotype in MS, which is also observed in an experimental model of de- and remyelination\textsuperscript{41}. These observations, and that remyelination is more efficient in GM than in WM\textsuperscript{17–19,37,38}, are an indication of the significance of differences in macroglia from different brain regions for remyelination. A more refined approach taking regional differences in macroglia into account will be beneficial for myelin research, and for the identification of therapeutic targets that may promote remyelination, particularly in WM lesions where remyelination is less effective.