6

General Discussion

1. Background

The importance of glial cells in health and disease has received increasing attention and appreciation over the past few decades. For example, glia have been found essential for axonal conduction, they regulate synapse formation, and control synaptic strength. Nevertheless, we have much to learn about the specific mechanisms by which glial cells contribute to brain functioning. In particular, much knowledge is lacking on the genetic contribution of glial cells to polygenic disorders such as psychiatric diseases.

In this thesis, we focused on glial genetics to gain more insights into roles of glial factors in CNS connectivity and psychiatric diseases. We have utilized a multidisciplinary approach, involving human and mouse experimental data, and consisting of a combination of bioinformatics, systems biology, wet lab techniques, and *in vivo* transgenic analyses. This allowed us to identify specific glial functions that might underlie primary processes in CNS diseases and disorders. We hope our findings will contribute to acknowledging pervasiveness of glial cells in brain pathology and diseases, and extension of targets for medicinal research beyond solely neurons and synapses.

2. Association of glial genetic variants with psychiatric diseases in humans

In chapters 2 and 3 of this thesis, we set out to investigate association between psychiatric diseases and variations in groups of functionally related glial genes. For this purpose, we created lists of non-overlapping genes for astrocytes, oligodendrocytes and microglia, and applied functional gene set analysis on human single nucleotide polymorphism (SNP) data to identify glial genetic variants in schizophrenia (chapter 2), and Tourette syndrome (chapter 3).

For schizophrenia, we found associations with several astrocyte and oligodendrocyte gene sets, but no association with any of the microglia gene sets. Our results seem to

be in line with current theories on schizophrenia [1-12]. The significantly associated astrocyte and oligodendrocyte gene transcription sets consist of many factors known to play important roles in glial developmental processes, and indicate that both oligodendrocytes and astrocytes may be viewed as important contributors to schizophrenia in the context of a neurodevelopmental disease [1-3]. Our astrocyte findings may shed new light on theories of synapse dysfunction in schizophrenia [2-7], since astrocyte genes involved in regulation of synapse function were found. Our findings of oligodendrocyte mechanisms in schizophrenia follow reported white matter alterations in schizophrenia [8-12], and suggest genetic defects in oligodendrocyte lipid metabolism genes that may relate to myelin membrane integrity. Interestingly, we recently performed a follow up on a small independent cohort (77 subjects with schizophrenia and 104 controls) in which association of white matter integrity and oligodendrocyte genes in schizophrenia was analyzed [13]. Indeed, fractional anisotropy analysis showed abnormalities in global white matter integrity in schizophrenia that associated with an oligodendrocyte myelination-related gene set. Comparison with our findings in Goudriaan et al. [14] (i.e. chapter 2) is complicated due to different grouping of the oligodendrocyte-enriched genes in functional gene sets, with seven sets in Chavarria-Siles et al. [13] versus 29 sets in Goudriaan et al. [14]. Nevertheless, together the two studies suggest that genetic variants in oligodendrocyte lipid and myelin genes may affect white matter integrity, and thereby increase the risk for schizophrenia. We hypothesize that disrupted myelin might affect long-distance signaling in the brain, leading to disrupted integration of signals from distant brain regions. The possible implications of myelin and glial lipids in CNS functioning and connectivity will be discussed in more details below (paragraphs 3 and 4).

For Tourette syndrome, we found a significant association with only one astrocytic subset of 33 genes involved in glycolysis and glutamate metabolism, through which astrocytes are thought to support synaptic function. Importantly, no results were found for any of the neuronal synaptic gene sets that were taken along in the analysis,

as previously reported by Lips et al. [15]. Although genetic factors play an important role in the etiology of Tourette syndrome, genetic and molecular pathophysiologies of this disease are still largely unknown [16-18]. Our results indicate for the first time that the process of astrocyte-neuron metabolic support may be an important contributor to Tourette syndrome pathogenesis.

In addition to the two chapters on schizophrenia and Tourette syndrome, our lists have recently also been used to identify glial genetic variants associated with normal cognitive performance, as measured by educational attainment (Polderman et al., personal communication). Significant associations in both glial functioning, and neuronal synaptic functioning were found. For glia, the most significant association was found for oligodendrocyte genes involved in oxidation reduction. Changes in oxidation reduction can interfere with lipid metabolic processes and/or lead to generation of free radicals as reactive oxygen species (ROS) that can damage the myelin sheath via lipid peroxidation. Even though the focus of this thesis is on psychiatric diseases, this finding suggests that altered myelin lipid metabolism might also contribute to variation in normal cognitive performance.

Taken together, the creation of our glial gene lists has provided a new tool for investigation of glial genetics in human brain functioning, and has led to the opportunity of creating new insights into the role of glial genetics in different human mental illnesses. Moreover, the lists may be applied for studying variation in cognitive traits as well. For in depth-studied diseases, such as schizophrenia, our results are well in line with other current theories, thus indicating validity of this tool. In addition, we have found exciting results for diseases and processes for which little of the underlying genetic and molecular mechanisms are yet known. Interestingly, recurrent findings with our lists for different diseases and processes include astrocyte regulation or support of synapse functioning, and involvement of oligodendrocyte lipid or myelin genes that likely influence integrity of the myelin membrane sheath. This underscores

that genetic variation in these processes might have widespread effects on brain physiology and behavior.

Despite this promising use for future studies, there are a number of issues to be considered: first, for the generation of our lists, we have used elaborate filtering steps to enhance association of the genes with specific cell-types. For instance, overlap between all of the glial lists and furthermore with a neuronal list was removed. Nevertheless, some of the genes in the significant glial gene sets might still have important roles in neurons or the other glial cell-types, and it cannot be excluded that these contribute to the studied phenotypes as well. Frequent updates and improvements in the curation of the lists should help tackle this issue to a certain extent, but subsequent experimental research will also remain crucial for further validation and insights of the obtained glial results. For example, manipulation of astrocytic carbohydrate metabolism might represent an exciting new opportunity for design of new cellular and/or animal models for Tourette syndrome, and a first step towards future medicinal research. Second, we mainly focused our investigations on glial functions, and used our neuronal list, based on the same literature and data as the glial lists, for filtering purposes only. Nevertheless, for comparative reasons it might be better to include neuronal gene sets in the analyses as well. For instance, synaptic gene sets created by Lips et al. [15] were included for our analysis on Tourette syndrome (chapter 2). However, none of these sets reached genome wide significance. This might have important consequences for future therapeutic research, as it seems that only the perisynaptic astrocyte should be considered as a cellular target. Nevertheless, the synaptic sets by Lips et al. [15] were constructed using different methods than our microarray-based, literature curated lists. By performing GO-analyses and expert curation of our own neuronal list into functional gene sets as well, a proper comparison between gene sets for glia versus neurons would be possible. Third, the literature on microglial expressed genes contained much smaller numbers than for the two macroglia cell-types, and were furthermore mainly focusing on analysis of the activated microglia cell-type [19-25]. Our final set of microglia genes might thus not

encompass all microglia functions. Specifically, possible contributions of genes and functions associated with the quiescent phenotype might have been overlooked in the current analyses. During writing of this thesis, several papers have appeared describing microglia gene expression for larger number of genes, and both for active, as well as quiescent microglia [26-28]. Including these data to revise our glia gene lists is expected to improve their quality and the strength of future findings using these lists. Finally, our association analyses thus far have been applied using human SNP data only. Nevertheless, there has been an increasing awareness for involvement of other type of genetic variants (e.g. microdeletions or duplications) in psychiatric diseases, such as schizophrenia [29, 30]. It would be interesting to repeat analyses with our glial lists and investigate association of rare genetic glial variants as well.

3. Genetics underlying differences in oligodendrocytes and myelination in health and disease

Myelin abnormalities have been implicated in a wide variety of mental disorders, such as schizophrenia, autism, depression, drug addiction and Alzheimer's disease [31-36]. Moreover, one of our recurrent findings in the functional gene set analysis was represented by mutations in groups of oligodendrocyte lipid and myelin genes related to the structural myelin sheath. Associations were found for schizophrenia in two independent cohorts of patients [13, 14], as well as for variation in normal cognitive functioning (Polderman et al., personal communication).

In order to further delve into molecular, cellular and functional consequences of variation in oligodendrocyte and myelin genes, we moved from human SNP data to experimental mouse models (chapters 4 & 5). We reasoned in chapter 4, that genetic diversity in recombinant inbred (RI) mouse strains may provide a good opportunity for studying molecular and functional (e.g. connectivity and/or behavior) correlates of variation in oligodendrocyte and myelin related genes. A major advantage of the BXD

RI strains, is that sequence data, experimental expression data, and phenotype data for many complex traits is publicly available [37-43].

Using a database of microarray expression data, we found strong differences in myelin gene expression between the BXD RI strains that correlated with differences in anxiety/activity behavioral traits. Accordingly, the parental strains C57BL/6J (C57) and DBA/2J (DBA) showed differences in myelin protein composition, myelin/axonal structure, and CNS conduction velocity. Several studies have emerged that point at conduction velocity and processing speed as predictors of general intelligence [44, 45], or that report correlations between processing speed and cognitive impairments for patients with multiple sclerosis [46]. We thus hypothesize that our myelin-related findings may (partly) underlie previously reported behavioral differences between C57 and DBA mice [47-49].

On a transcript level, all myelin genes investigated showed similar regulation over the different BXD lines, i.e. if a BXD strain showed a higher or lower expression of a myelin gene compared to the mean of this gene for all BXD strains combined, this would in general be seen for all other myelin genes as well. This observation is in line with many published studies indicating presence of transcription factors that specifically regulate expression of groups of oligodendrocyte and myelin genes [50-55]. The robust differences we found between BXD RI strains in myelin gene transcription were, however, on the protein level only reflected for a subset (e.g. MAG, MOBP, CNP and NCAM). This is not uncommon considering the many regulatory processes that occur in-between gene transcription and protein expression [56]. Nevertheless, this finding might have important implications for further studies, as this indicates that differences in myelin gene expression levels do not simply translate to more or less myelin in the brain. Instead, the effects seem more subtle, specifically influencing composition of the myelin sheath, and possibly relate to how certain myelin molecules interact with axonal components.

We propose that C57 and DBA mice might represent interesting models to further investigate how genetic variation in myelin genes might influence brain connectivity, and contribute to behavioral phenotypes. Especially MAG, with its differential protein expression between C57 and DBA for its L- and S-isoforms, poses as an interesting candidate. The L-MAG protein expression difference between C57 and DBA was a striking, almost 10-fold higher expression in brain and purified myelin of DBA mice, whereas total MAG was higher expressed in C57 mice (i.e. indicating higher expression of S-MAG). Interestingly, the total amount of MAG is much higher in CNS compared to PNS [57]. Moreover, L-MAG is expressed in the adult CNS, but not in the adult PNS [57, 58]. The substantially higher concentration of MAG in the CNS may reflect the need for a larger amount of this glycoprotein for signaling at the oligodendroglial–axon junction than at the Schwann cell–axon junction [57], and indicates an involvement of MAG in higher brain functions. Accordingly, behavioral studies on MAG-null mice indicate functional cognitive and/or locomotor deficits [57]; and MAG has been implicated in pathology associated with psychiatric diseases as schizophrenia [57, 59-63], and neurological disorders as multiple sclerosis [57, 64-66]. Interestingly, human MAG was also one of the genes in our myelination-related gene set for which Chavarria-Siles et al. [13] recently reported association with perturbed white matter integrity in schizophrenia patients. Importantly, MAG knockout mice, like DBA, have smaller diameter axons with thinner myelin [67, 68]. It has been proposed that S-MAG is mainly involved in axonal signaling and modulating axonal caliber through phosphorylation, and other axonal changes needed for maintenance and survival, whereas the L-isoform is especially important for signaling in the opposite direction, and enhancing the capacity of oligodendrocytes to form and maintain myelin [57]. In line with this, immunohistological experiments have shown that L-MAG is associated with smaller oligodendrocytes that myelinate numerous relatively small axons, whereas S-MAG is associated with larger type oligodendrocytes, that myelinate only a few larger axons [57, 69]. It can thus be hypothesized that the higher expression of L-MAG in DBA

contributes to the smaller myelinated axons, and consequently slower conduction velocities of myelinated axons, in DBA compared to C57.

It has been shown that during early stages of CNS myelination the L-MAG isoform is predominant, while in the adult CNS both L- and S-MAG are present, at approximately equal amounts [57]. As mentioned above, this is in sharp contrast with the adult PNS, where L-MAG is absent, and only S-MAG expressed [57, 58]. Based on these observations, we suggest that L-MAG might be important for the initial stages of myelination, and S-MAG for maturation and maintenance of myelinated axons. The absence of L-MAG in PNS of adult rodents might relate to the process of myelination and axonal maturation being done after postnatal development; whereas L-MAG in CNS of adults might be associated with the fact that myelination and axonal maturation in the brain is a plastic process that continues well into adulthood.

There are several mouse models for compromised MAG expression, including two lines of transgenic MAG-null mice [70, 71], a transgenic mouse model of truncated L-MAG where only S-MAG is expressed [72], and quaking mice, where a recessive mutation affects CNS myelination, and L-MAG expression is severely reduced [73-75]. To our knowledge, DBA mice might represent the first rodent model for relative overexpression of L-MAG. The specific processes that affect MAG splicing in such a way that it mimics the expression patterns for L- and S-MAG as seen in C57 and DBA are not yet deciphered. When comparing MAG gene structure between C57 and DBA via the GeneNetwork database, many SNPs are revealed. As much as four Non-Synonymous SNPs are present in exons seven, 11 and 12 (table 1). Markedly, two of these SNPs are located within exon 12 that is specific for the S-MAG isoform. One could speculate that these might affect total MAG and/or S-MAG expression in DBA; and that consequently the higher levels of L-MAG in DBA might represent a compensatory process. Nevertheless, it remains to be determined whether the differences in MAG protein expression in C57 and DBA are related to 1) a common factor influencing mRNA expression of multiple myelin genes as a set, 2) genetic variants in upstream MAG

regulatory or splicing factors, or 3) these specific genetic variants in MAG gene structure itself that might influence MAG protein structure, functioning, or it's interaction with upstream regulatory elements. Using molecular cloning techniques it would be possible to further identify specific effects of genetic variants between C57 and DBA on MAG functioning and structure by investigating expression of genetically engineered vectors or plasmids in primary oligodendrocyte cultures, or primary co-cultures of oligodendrocytes and neurons. Effects of SNPs in the MAG gene could be investigated using cells derived from MAG null-mice. Eventually, such experiments might even be used as a basis for transgene targeting in mice and subsequent electrophysiology, myelin connectivity and behavioral experiments, to further investigate causality of the here reported findings.

On another note, our findings on large differences in myelin gene and protein expression between RI mouse strains, warrants precaution when investigating mouse models of demyelinating disorders. For example, mouse models of multiple sclerosis such as experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalitis virus-induced demyelinating disease (TMEV-IDD). It has been well documented that the ease of disease induction, protocols for disease induction, as well as disease progression, symptoms, and experimental outcomes, differ per mouse strain [76-78]. In essence, multiple sclerosis, EAE and TMVED-IDD are characterized as inflammatory disorders [77, 78]. Recent studies are showing links between myelin defects and inflammation [79], and axonal damage and secondary inflammation have been observed in several transgenic models for myelin proteins, including those for which we observed expression differences. For example, in CNP1 mutants neuroinflammation in white matter tracts is significantly enhanced after minor brain injury [80], and crush-injured nerves of MAG-deficient mice show impaired macrophage efflux from Schwann cell basal lamina containing myelinated axons [81]. Possible influences of genetic variation on myelination and myelin protein expression might thus be of relevance when studying inflammatory, demyelinating diseases as multiple sclerosis and potentially new therapeutic targets.

Table 1. SNPs between DBA and C57 in structure of Mag gene

SNP ID	Chr	Mb	Alleles	Source	Con Score	Gene	Transcript	Exon	Domain 1	Domain 2	Function	Details	C57BL/6J	DBA/2J
wt37-7-31684270	7	31,68427	G/A	Sanger/UCLA	1	Mag	ENSMUST00000040548		Exon	3' UTR			G	G
MRS1995948	7	31,68436	A/C	UTHSC_CITG	1	Mag	ENSMUST00000040548		Exon	3' UTR			A	C
wt37-7-31684406	7	31,68441	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548		Exon	3' UTR			C	C
wt37-7-31684448	7	31,68445	G/A	Sanger/UCLA	1	Mag	ENSMUST00000040548		Exon	3' UTR			G	G
wt37-7-31684532	7	31,68453	G/C	Sanger/UCLA	1	Mag	ENSMUST00000040548		Exon	3' UTR			G	G
wt37-7-31684544	7	31,68454	G/A	Sanger/UCLA	1	Mag	ENSMUST00000040548		Exon	3' UTR			G	A
wt37-7-31684663	**7**	**31,68466**	**A/G**	**Sanger/UCLA**	**1**	**Mag**	**ENSMUST00000073818**	**12**	**Exon 12**	**Coding**	**Nonsynonymous**	**Biotype: Protein Coding, S -> P, Tcc -> Ccc, 591**	A	G
wt37-7-31684714	**7**	**31,68471**	**C/T**	**Sanger/UCLA**	**1**	**Mag**	**ENSMUST00000073818**	**12**	**Exon 12**	**Coding**	**Nonsynonymous**	**Biotype: Protein Coding, E -> K, Gag -> Aag, 574**	C	T
wt37-7-31685675	7	31,68568	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	11	Exon 11	Coding	Synonymous	Biotype: Protein Coding, G -> G, ggG -> ggA, 566	C	T
wt37-7-31685693	7	31,68569	G/A	Sanger/UCLA	1	Mag	ENSMUST00000040548	11	Exon 11	Coding	Synonymous	Biotype: Protein Coding, P -> P, ccC -> ccT, 560	G	A
wt37-7-31685696	7	31,6857	G/A	Sanger/UCLA	1	Mag	ENSMUST00000040548	11	Exon 11	Coding	Synonymous	Biotype: Protein Coding, S -> S, agC -> agT, 559	G	G
wt37-7-31685720	7	31,68572	T/A	Sanger/UCLA	1	Mag	ENSMUST00000040548	11	Exon 11	Coding	Synonymous	Biotype: Protein Coding, G -> G, ggA -> ggT, 551	T	T
wt37-7-31685737	**7**	**31,68574**	**A/G**	**Sanger/UCLA**	**1**	**Mag**	**ENSMUST00000040548**	**11**	**Exon 11**	**Coding**	**Nonsynonymous**	**Biotype: Protein Coding, S -> P, Tcc -> Ccc, 546**	A	G
wt37-7-31685744	7	31,68574	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	11	Exon 11	Coding	Synonymous	Biotype: Protein Coding, T -> T, acG -> acA, 543	C	T
wt37-7-31685750	7	31,68575	A/G	Sanger/UCLA	1	Mag	ENSMUST00000040548	11	Exon 11	Coding	Synonymous	Biotype: Protein Coding, N -> N, aaT -> aaC, 541	A	G
wt37-7-31686732	7	31,68673	G/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	*9*	Exon 9	Coding	Synonymous	Biotype: Protein Coding, R -> R, cgC -> cgA, 485	G	G

SNP ID	Chr	Mb	Alleles	Source	Con Score	Gene	Transcript	Exon	Domain 1	Domain 2	Function	Details	C57BL/6J	DBA/2J
wt37-7-31686819	7	31,68682	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	9	Exon 9	Coding	Synonymous	Biotype: Protein Coding, T -> T, acG -> acA, 456	C	C
wt37-7-31691926	7	31,69193	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	8	Exon 8	Coding	Synonymous	Biotype: Protein Coding, E -> E, gaG -> gaA, 410	C	C
wt37-7-31692154	7	31,69215	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	8	Exon 8	Coding	Synonymous	Biotype: Protein Coding, T -> T, acG -> acA, 334	C	C
wt37-7-31693418	7	31,69342	G/C	Sanger/UCLA	1	Mag	ENSMUST00000040548	7	Exon 7	Coding	Synonymous	Biotype: Protein Coding, G -> G, ggC -> ggG, 312	G	G
wt37-7-31693498	**7**	**31,69 35**	**T/C**	**Sanger/ UCLA**	**1**	**Mag**	**ENSMUST00000040548**	**7**	**Exon 7**	**Coding**	**Nonsynonymous**	**Biotype: Protein Coding, K -> E, Aag -> Gag, 286**	T	C
wt37-7-31693512	7	31,69351	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	7	Exon 7	Coding	Nonsynonymous	Biotype: Protein Coding, R -> K, aGg -> aAg, 281	C	C
wt37-7-31694007	7	31,69401	A/G	Sanger/UCLA	1	Mag	ENSMUST00000040548	6	Exon 6	Coding	Synonymous	Biotype: Protein Coding, L -> L, Ttg -> Ctg, 234	A	G
wt37-7-31694074	7	31,69407	G/A	Sanger/UCLA	1	Mag	ENSMUST00000040548	6	Exon 6	Coding	Synonymous	Biotype: Protein Coding, N -> N, aaC -> aaT, 211	G	G
wt37-7-31694089	7	31,69409	A/G	Sanger/UCLA	1	Mag	ENSMUST00000040548	6	Exon 6	Coding	Synonymous	Biotype: Protein Coding, P -> P, ccT -> ccC, 206	A	G
wt37-7-31696664	7	31,69666	G/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	5	Exon 5	Coding	Synonymous	Biotype: Protein Coding, G -> G, ggC -> ggA, 57	G	G
wt37-7-31696679	7	31,69668	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	5	Exon 5	Coding	Synonymous	Biotype: Protein Coding, P -> P, ccG -> ccA, 52	C	C
wt37-7-31696700	7	31,6967	G/A	Sanger/UCLA	1	Mag	ENSMUST00000040548	5	Exon 5	Coding	Synonymous	Biotype: Protein Coding, D -> D, gaC -> gaT, 45	G	G
wt37-7-31696979	7	31,69698	A/G	Sanger/UCLA	1	Mag	ENSMUST00000040548	4	Exon 4	5' UTR			A	A
wt37-7-31699302	7	31,6993	G/T	Sanger/UCLA	0,196	Mag	ENSMUST00000040548	2	Exon 2	5' UTR			G	G
wt37-7-31699324	7	31,69932	A/T	Sanger/UCLA	0,196	Mag	ENSMUST00000040548	2	Exon 2	5' UTR			A	A
wt37-7-31699843	7	31,69984	C/T	Sanger/UCLA	1	Mag	ENSMUST00000040548	1	Exon 1	5' UTR			C	T
wt37-7-31699850	7	31,69985	A/G	Sanger/UCLA	1	Mag	ENSMUST00000040548	1	Exon 1	5' UTR			A	G

Table 1: *legend on next page*

Table 1: Data derived from GeneNetwork database (http://www.genenetwork.org/webqtl/main.py). Nonsynonymous SNPs are shown **bold** and highlighted. For SNPs in non-coding intron regions please refer to SNP analyser on GeneNetwork.

4. Lipid metabolism as determinant for myelin integrity and connectivity

The myelin membrane stands out for its high lipid content. Lipids account for at least 70% of the dry weight [82], which is twice that of other plasma membranes [83]. This makes the myelin membrane vulnerable for lipid metabolism disorders. As shown with our glial gene lists, defects or variation in glial lipid metabolism related to the myelin membrane sheath might contribute to psychiatric diseases and differences in cognition. In chapter 5 of this thesis, we further focused on specifically the contribution of glial lipids to myelin membrane synthesis in the CNS.

Myelination is mostly dependent on lipid synthesis by myelinating glia, without contribution of exogenous lipids [83]. Nevertheless, two recent studies, one on Schwann cells in the PNS [84], and the other on oligodendrocytes in the CNS [85], showed that genetic impairment of lipid metabolism interferes with the acute phase of myelination, but also that uptake of extracellular lipids may partially rescue myelination. Here, we explored the contribution of extracellular lipids to myelination by oligodendrocytes, and their cellular origin. This was done with specific focus on astrocytes as these cells are highly active in synthesis and secretion of lipids [86-92], and are able to promote myelination *in vitro* in neuron-oligodendrocyte co-cultures [93-95].

By genetically interfering with lipid metabolism via SCAP deletion in oligodendrocytes (CNP-SCAP) and/or astrocytes (GFAP-SCAP) *in vivo*, we found that myelin membrane synthesis by oligodendrocytes requires both intrinsic lipid biosynthesis, as well as the uptake of extracellular lipids that seems to be mainly provided by astrocytes; and which becomes most limiting in later phases of myelination. In addition, we showed that when astrocyte lipid biosynthesis is compromised, oligodendrocytes incorporate circulating extracellular lipids in the myelin membrane, and that a lipid-enriched diet

partly rescues myelination and conduction velocity of myelinated axons. The virtual absence of myelin around each axon when SCAP is deleted in both oligodendrocytes and astrocytes shows that these two cell types are the main lipid contributors for the oligodendrocyte myelin membrane.

We propose that endogenous lipid levels in oligodendrocytes are sufficient for initial myelin membrane synthesis in the first postnatal weeks, while subsequent elaboration of a full myelin membrane requires lipid supply from astrocytes. Inducible astrocyte-specific and oligodendrocyte-specific KO mice [96-98] may be used to further investigate the effects of SCAP KO during different stages of myelination in astrocytes and oligodendrocytes. An issue that warrants further investigation is related to a recent finding that GFAP is not only expressed in astrocytes, but during development also in progenitor cells giving rise to neurons, oligodendrocytes and astrocytes [98]. We can therefore not exclude that part of our findings in the GFAP-SCAP KO may also be due to compromised lipid metabolism in non-astrocyte cells. Primary cultures of astrocytes, neurons and oligodendrocytes may be used to directly investigate effects of compromised lipid metabolism in isolated astrocytes from GFAP-SCAP KO on myelination of WT neurons and oligodendrocytes. Inducible KO strategies may also be used to circumvent this problem: by deleting SCAP using inducible Cre driven by GFAP or GLAST promoters at later developmental stages, SCAP KO will be more exclusively restricted to astrocytes [98].

We believe our data provides a new perspective for a broad spectrum of diseases where lipid metabolism may be affected or plays a role. This not only applies to diseases with a clear myelin phenotype (e,g, Smith-Lemli-Opitz syndrome, Sjogren-Larsson syndrome, Refsum disease, adrenoleukodystrophy, and multiple sclerosis [83]), but also to other CNS diseases in which deregulated lipid metabolism and myelin defects have been implicated (e.g. schizophrenia, bipolar disorder, Parkinson's, Alzheimer's and Niemann-Pick [99-104]). Roles for glial lipid metabolism in psychiatric diseases and cognition are supported by our functional gene set analysis in which we

found functional associations between oligodendrocyte lipid metabolism and schizophrenia [14], and between oligodendrocyte genes involved in oxidation reduction (mostly related to lipid metabolism) and educational attainment (Polderman et al., personal communication). So far, we did not find links between astrocyte lipid gene sets and human diseases or cognition. Nevertheless, such a set might lack specificity for genetic associations related to myelin membrane biogenesis, as astrocytic lipids are involved in multiple brain functions and glia-neuron interactions [86, 87]. Together, these studies are linking genetic variation in myelin lipid metabolism with cognitive (dys)function and vulnerability to psychiatric diseases.

Recently, ketogenic diets, which are high in fat and low in carbohydrates, were shown to be efficient for treatment of a wide range of neurological disorders [105, 106], including treatment of refractory epilepsy in patients and mice [107], decreasing beta-amyloid depositions in transgenic models for Alzheimer's disease [108], improving motor deficits in a transgenic model of amyotrophic lateral sclerosis [109], and ameliorating motor deficits as well as contributing positively to synaptic plasticity, learning and memory, in the murine EAE model of multiple sclerosis [110]. In addition, it has been shown that antipsychotic medication upregulates expression of genes that are involved in cellular fatty acid and cholesterol biosynthesis under control of the SREBP transcription factors [100, 111]. These studies are in line with our results showing that deficient endogenous lipid synthesis and perturbed lipid metabolism cause disabling defects in the nervous system, and that these deficits can be directly treated by lipid supplementation in the diet [87, 106].

It should be noted that in a previous mouse model of compromised cholesterol metabolism in oligodendrocytes (CNP-SQS) horizontal cholesterol transfer was suggested to improve myelination over time as well [85]. However, a cholesterol-enriched diet did not improve myelination in these mice. Because oligodendrocytes do not have the same access to circulating lipids as astrocytes, we propose that the beneficiary effects of the lipid diet in the GFAP-SCAP mice may involve the close vicinity

of astrocyte end-feet to blood capillaries, and thereby the uptake of circulating lipids by astrocytes and subsequent delivery of lipids to oligodendrocytes. In order to test this hypothesis, it would be preferred to investigate the effects of the lipid diet in our oligodendrocyte lipid compromised mice (CNP-SCAP) as well, and see whether the results are comparable to the CNP-SQS mice. Secondly, our hypothesis implies that diet-derived-lipids can cross the blood-brain barrier. This is an unexpected finding, as it is generally assumed that circulating lipids are largely excluded by the blood-brain barrier [99]. Various compensatory mechanisms, however, have been identified in CNS models of compromised brain cholesterol homeostasis, which might elevate lipid transport across the blood-brain barrier. These include an increased angiogenesis of brain capillaries [112, 113], and increased expression of lipoprotein receptors in brain capillary cells [112, 114]. To our knowledge, *in vivo* methods with validated success for imaging and measuring crossing of lipids from diet into the brain are not yet available. Nonetheless, the blood-brain barrier can be modeled *in vitro* using primary co-cultures of brain endothelial cells and astrocytes [115-117]. Such cultures may be used for additional insights in crossing of dietary derived lipids to the brain by investigating blood-brain barrier characteristics (e.g. permeability, receptors, transporters) from our SCAP KO animals under naive and high fat diet conditions. Indeed, it was recently shown via several *in vitro* models of this barrier that endothelial cells might cooperate with astrocytes to allow crossing of n-3 fatty acid precursors and provide these to astrocytes [88]. In addition, it might be interesting to see whether a recent study design in which drug delivery across the blood-brain barrier was studied *in vitro* using a reporter vector, can be adapted for studying delivery of lipid transport *in vitro* across the blood-brain-barrier as well [118].

5. Conclusions and perspectives

In this thesis, we utilized three different genetic approaches to investigate glial factors contributing to brain functioning and behavior. We started with a focus on all glial cells

and functions in humans, and pursued more in-depth analysis focusing specifically on glial genetics in myelin membrane integrity and functioning in mouse models. 1) Thus far, our newly developed glial gene sets have indicated involvement of astrocyte genes related to synapse functioning, and oligodendrocyte myelin and lipid genes related to the structural myelin membrane sheath, in human psychiatric diseases and cognition. 2) Studying genetic variation in myelin genes and proteins in BXD mice, we found correlations between myelin genes and anxiety/activity related behavior, differences in protein composition of the myelin sheath, and differences in speed of compound action potentials in myelinated axons of the CNS. 3) Using transgenic mouse models for compromised lipid metabolism, we showed the importance of glial lipids for myelination and neuronal network connectivity. In addition, high fat diet may partially rescue myelin deficits due to compromised glial lipid metabolism.

The involvement of astrocyte genes in human psychiatric diseases was mostly driven by genes involved in synapse functioning. As astrocytes can contact up to thousands of synapses, we propose that astrocytes may participate in information processing by coordinating synaptic activity among sets of neurons. As myelinated axons travel long distances in the CNS, interactions of the myelin membrane sheath with neuronal axons have profound influence on long-distance signaling in the brain. By regulating the speed of transmission in axons of different lengths, myelin might influence synchronous arrival in post-synaptic targets. Both astrocytic coordination of synapse activity, and myelin regulation of conduction velocity speed could have important consequences on integration and processing of signals from different brain regions, and consequently, behavior. With lipids representing important constituents of the myelin sheath, high fat lipid diets should be considered as therapeutic targets in a variety of neurological and psychiatric disorders in which glial lipid metabolism might be affected or plays a role.

We feel these results are exciting, indicating that glial genes are key candidates underlying brain physiological and behavioral deficits. This may have important

implications for the understanding and treatment of neurological and psychiatric diseases.

6. Recommendations for further studies

This thesis gives insights into a variety of glial genetic factors in health and disease, but also gives rise to ample experiments to further our understanding of the above-proposed model. We hypothesized that compromised glial lipid metabolism potentially contributes to differences in human behavior and psychiatric diseases, which is supported by genetic associations found with our glial gene lists. However, behavioral studies to investigate direct links between compromised glial lipid metabolism and behavior in our transgenic SCAP models are complicated: 1) In GFAP-SCAP KO mice, defects in myelination are but one of many disturbed CNS processes [87]; and 2) in CNP-SCAP KO mice behavioral studies are compromised by the severe effects on motor and sensory functioning. Inducible SCAP KO mice might help to tackle this problem, as severity of compromised lipid metabolism in oligodendrocytes might be related to the developmental stage of myelination. Transgenic mice with more subtle effects on myelination might be more suitable for use in behavioral studies; and thereby showing a direct link between myelin membrane lipids and behavior.

Also, we have mainly collected and interpreted our data with a focus on roles of myelin in network connectivity via regulation of the speed of action potentials. It has recently become clear however, that myelin also plays an important role in the support of long-term integrity and survival of axons [79, 119]. It remains to be determined whether the here reported myelin and oligodendrocyte related findings might also contribute to differences in brain wiring and behavior via regulation of neuronal integrity and trophic support of axons.

An exciting hypothesis states that myelin might be modifiable by experience [120]. It would be interesting to see if the here reported findings might also play roles in these

so-called myelin plasticity processes [120]. MAG might be an interesting candidate, as MAG is already known for playing a role in formation of synaptic circuits during development, by inhibiting sprouting of axons and influencing critical periods of synaptic plasticity [120, 121]. In paragraph 3, we postulated a hypothesis stating that L-MAG in CNS might be involved in processes of initial myelination continuing well into adulthood, whereas S-MAG might be expressed by maturated, myelinated axons. It would be interesting to see if L/S-MAG ratios in CNS myelin might be influenceable by neuronal activity. In particular, we propose that by stimulating maturation of ensheathed axons, neuronal activity might ultimately increase expression of S-MAG in the CNS in C57 mice. Considering the higher L/S-ratio's in DBA, we hypothesize that these mice might show less, or compromised, regulation of MAG splicing as a result of neuronal activity, and that these mice might compensate via increased expression of L-MAG.

As we have shown that glial lipids are rate limiting for CNS myelination, the associations we reported for oligodendrocyte lipid metabolism with psychiatric diseases and cognition, might also be (partly) explained by compromised lipid metabolism interfering with myelination in response to neuronal activity and experience. Further insights into roles of here investigated myelin and glial lipid genes in myelin plasticity processes [120], might be derived by *in vitro* as well as *in vivo* approaches. *In vitro*, co-cultures of oligodendrocytes and neurons could be used to study influences of neuronal activity on myelination, and expression of lipid and myelin proteins. By using oligodendrocytes from MAG or SCAP transgenic mice, or by targeting MAG or SCAP functioning in oligodendrocytes via siRNA vectors or plasmids, direct influences of neuronal activity on myelination via oligodendrocyte lipid or MAG mediation, can be investigated. *In vivo*, correlations between experiences (f.e. enriched vs. non-enriched environment), axonal/white matter structure, conduction velocity speed, and myelin lipid composition or MAG expression may be studied. These investigations can be made more causal, by comparing WT with transgenic SCAP mice, mouse models for

compromised MAG expression [70-75, 122], and by comparing effects in C57 vs. DBA mice.

Surprisingly, the deficits due to compromised glial lipid metabolism could be directly treated by lipid supplementation in the diet. We believe our data provides a new perspective for a broad spectrum of diseases where lipid metabolism may be affected or plays a role, including psychiatric diseases, such as schizophrenia. Nevertheless, effects of dietary supplementation in patients are not consequent and with mixed results [123, 124]. Moreover, the high fat diet in our transgenic mice only partially rescued myelination and conduction velocities, and the exact mechanisms by which the high fat diet improved myelination remains to be determined. This calls for optimization of diets in terms of their composition and timely application, and further experiments in order to obtain a better understanding of potential, underlying mechanisms.

Finally, the human SNP data was used to obtain insights into processes that may underlie human psychiatric diseases, and mouse models were used to further investigate molecular and cellular mechanisms. A relatively new method for differentiation of human fibroblasts into induced pluripotent stem cells (IPSC) [125-127], could be used to complement our molecular and cellular results with human data as well. With this technology, isolated cells from human patients can be differentiated towards glial cells, after which genetically compromised functions may be determined *in vitro* (co-cultures) and *in vivo* (mouse cell transplantations). These techniques could be utilized to search for more causal evidence of involvement of the glial genes and functions we identified with functional gene set analysis, but also to investigate whether perturbed MAG and glial lipid metabolism as investigated in chapters 4 & 5 can be directly linked to human disease.

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