Genetic Variation in CNS Myelination and Functional Brain Connectivity in Recombinant Inbred Mice

Andrea Goudriaan, Maarten Loos, Sabine Spijker, August B Smit, Mark H.G. Verheijen
Abstract

Myelination greatly increases the speed of action potential propagation of neurons, thereby enhancing efficacy of inter-neuronal communication, and hence potentially, optimizing the brain’s signal processing capability. The impact of human genetic variation on the extent of axonal myelination and its consequences for brain functioning remain to be determined. Here we investigated this question using a genetic reference panel (GRP) of mouse BXD recombinant inbred (RI) strains, which partly model genetic diversity as observed in human populations, and which show substantial genetic differences in a variety of behaviors, including learning, memory and anxiety. We found coherent differences in expression of myelin-associated genes in brain tissue of RI strains of the BXD panel, with the largest differences in the hippocampus. The parental C57BL/6J (C57) and DBA/2J (DBA) strains were on opposite ends of the expression spectrum, with C57 showing higher myelin transcript expression compared with DBA. Our experiments showed accompanying differences between C57 and DBA in myelin protein composition, total myelin content, and white matter conduction velocity. Finally, the hippocampal myelin gene expression of the BXD strains correlated significantly with behavioral traits involving anxiety and/or activity. Taken together, our data indicate that genetic variation in myelin gene expression, translates to differences observed in myelination, axonal conduction speed, and possibly in anxiety/activity related behaviors.
Introduction

Axonal myelination increases action potential propagation. In particular, the myelin membrane forces the axonal action potential to be generated only at interruptions of non-myelinated areas of the axon, called nodes of Ranvier, resulting in a fast, saltatory movement of the action potential along the axon [1]. Formation of myelin around neuronal axons is a complex process, which in the central nervous system (CNS) involves oligodendrocytes that provide insulation by locally wrapping their cellular processes around axons [1]. Recent studies have suggested that specific modalities of myelin in the mammalian brain permit more complex forms of network integration [1, 2]. For instance, the distance between nodes, the nodal length, or the thickness of the myelin wrapping can have profound effects on transmission speed of action potentials and coincidence of firing onto postsynaptic neurons [1-3].

In accordance with myelin fundamentally changing the way neural impulses are generated and transmitted [1], loss of myelin integrity can lead to severe neurological symptoms, for example as observed in multiple sclerosis [4]. In peripheral neuropathy, sensory and motor deficits represent core symptoms, and this has attracted most of the attention in studies on myelin. Recently, myelin also gained interest as contributor to cognitive performance and behavior, including general intelligence via regulation of conduction velocities in the brain [5, 6]; and a wide range of psychiatric disorders, such as schizophrenia, autism, mood disorders, drug addiction and Alzheimer's disease [2, 7-18]. Several inherited myelin disorders have been identified with a Mendelian mode of inheritance. However, predisposition to complex traits and psychiatric diseases is often characterized by genetic heterogeneity and a high probability of complex gene-by-gene and gene-by-environment interactions. To what extent variation in the expression of myelin genes contributes to complex behavioral traits and diseases remains to be determined.

Genetic reference populations (GRPs) in animals, for instance panels of recombinant inbred (RI strains, have been assembled to model, at least in part, the genetic
complexity in human populations, while enabling tight experimental control and allowing extensive replication studies [19-21]. The BXD mouse resource is currently one of the largest and best characterized mouse GRPs, composed of ~160 lines that descend from crosses and inbreeding of the parental lines C57BL/6J (C57) and DBA/2J (DBA) [19, 22]. A major advantage of BXD RI strains is that experimental expression data and phenotype data for many complex traits is publicly available, allowing for hypothesis generation [19, 23-29]. Genetic differences between C57 and DBA mice have been shown to translate into a broad spectrum of CNS related functional and molecular correlates, for example, differences in activity, impulsive action, hippocampal related memory and learning tasks, post- and pre-synaptic protein expression, and synaptic transmission and plasticity [30-42]. Through genetic linkage analyses, the genetic and phenotypic differences in the BXD panel of RI strains have resulted in identification of genes and loci involved in complex CNS functions, such as impulsivity [43], reversal learning [44], attention [45], neuronal oscillations [46] and hearing loss [47].

Possible differences in myelination between BXD RI strains, and the relation with their behavioral differences, have not yet been investigated. Here, we set out to investigate variation in myelin gene expression in the BXD GRP using expression data from the publicly available GeneNetwork database (www.genenetwork.org). We observed profound differences in expression of myelin genes over more than 70 BXD strains, correlating with behavioral phenotypes of activity and/or anxiety. The parental strains C57 and DBA were on opposite ends of the expression spectrum, with C57 showing higher myelin transcript expression. Furthermore, C57 and DBA showed differences in myelin protein content, myelin structure and white matter conduction velocity. We conclude that the differences in myelin composition might contribute to functional and/or behavioral differences in these strains. The BXD GRP might represent a promising genetic resource to further disentangle the molecular myelin substrates that influence variation in brain connectivity, and cognitive and behavioral traits.
Chapter 4: Genetic Variation in Myelination of RI Mice

Methods

Clustering of BXD gene expression data

Gene expression data from BXD mice strains was derived from the GeneNetwork database: [http://www.genenetwork.org/webqtl/main.py](http://www.genenetwork.org/webqtl/main.py). The GeneNetwork database provides open access to BXD and other RI strains derived microarray data, single nucleotide polymorphism (SNP) data, and phenotypic data for quantitative trait loci analysis and gene expression correlation analyses [23-28].

Gene expression data were exported for manually selected probes in the PDNN hippocampus database (Hippocampus Consortium M430v2 Jun06), and the PDNN whole brain database (INIA Brain mRNA M430 Jan06). The Hippocampus database was chosen as one of the most elaborate brain databases, as well as most highly recommended dataset on GeneNetwork itself ([http://www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_Accession Id=112](http://www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_Accession Id=112)). PDNN normalization was chosen, as this takes into account bias due to differential probe hybridization [28]. Analyses were performed including data for all BXD strains, parental C57 and DBA strains, and the two reciprocal F1 hybrids (N of strains is 71, pool size 2-4 mice per strain). Analyses were repeated with the PDNN whole brain database and were performed including data for BXD strains, and parental C57 and DBA (N of strains is 41, pool size 3 mice per strain).

Selected probes included those for myelin transcripts, as well as for transcripts involved in lipid metabolism and axonal/dendritic markers. Gene expression data was only included for probes fulfilling the following criteria: 1) 100% gene specificity as confirmed by Blast, 2) located on exons, and 3) with log2 expression values of >8, to prevent background noise in the data. The BXD expression data was scaled per probe by subtracting per probe, the total median of all BXD strains from the exported mean-value per strain. Thus, showing for that probe the deviation per strain in comparison to the median expression over all strains. If multiple probes per gene were present, the average for those probes was taken. Clustering was performed with TMEV.
software (http://www.tm4.org/mev.html), using Euclidean distance and average linkage clustering.

Animals

C57 and DBA were obtained from Charles River (Charles River Laboratories, L’Arbresle, France; European supplier of C57BL/6J mice, genetically indistinguishable from those obtained from The Jackson Laboratory [48]), and were housed with cage enrichment and water and food ad libitum on a 12/12 h rhythm lights on/off with lights on at 7 AM. All experiments were approved by the Animal Users Care Committee of the VU University and in compliance with the European Council Directive (86/609/EEC).

Preparation of total brain extract

Brains or hippocampi were freshly dissected on ice from C57 and DBA mice (postnatal day P20, and adult mice aged >8 to 12 weeks). Brains were separated from the spinal cord, medulla oblongata, and cerebellum. When hippocampi were dissected, the rest of the brain was kept and referred to as ‘rest of the brain’. Brain and hippocampi preparations were cleaned from the meninges, rapidly frozen in isopentane and/or on dry ice, and kept at −80 °C until used. On use, the tissue was homogenized in homogenization buffer consisting of 5 mM HEPES/NaOH, pH 7.4, 0.32 M sucrose, 0.016 U/mL of RNase inhibitor (Invitrogen, Carlsbad, CA, USA), complete EDTA-free, protease inhibitory cocktail (1 tablet/50 mL of homogenization buffer from Roche Applied Sciences, Indianapolis, IN, USA). This homogenate was used for all biochemical studies on tissue of hippocampus and whole brain.
RNA isolation, reverse transcription, and quantitative PCR

The mRNA from hippocampus and rest of the brain from C57 (n=6) and DBA (n=5) mice, aged 10 to 12 weeks, was isolated using TRIzol (Invitrogen), according to the manufacturer's protocol. Synthesis of cDNA and quantitative PCR (qPCR) reactions were performed as described previously [49]. The primer sequences are shown in table 1. Selection of housekeeping genes was performed according to Vandesompele et al. [50].

Immunoblotting on brain extracts

Whole brain homogenates (including hippocampus) were mixed with SDS sample buffer and heated to 90 ºC for 5 minutes. Proteins were separated by SDS-PAGE in a Mini-Protean electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) and electroblotted overnight onto polyvinylidene difluoride membranes. Membranes were probed with primary antibodies against the large isoform of MAG (L-MAG; Goat anti-mouse, 1:800, Santa Cruz), a Pan MAG antibody (P-MAG; recognizing L-MAG and small, i.e. S-MAG, isoforms; Mouse anti-mouse/rat/human, 1:1000, Abcam), MOBP (Rabbit antimouse/human, 1:500, Abcam), CNP (Mouse anti-mouse, 1:2000, Sigma),

Table 1: The sequences of primers used for qPCR analysis

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<td>VCP</td>
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MOG (Rabbit anti-mouse, 1:500, Sigma), MBP (Rat anti-mouse, 1:50, Abcam), Claudin11 (Rabbit anti-mouse/rat/human, 1:100, Santa Cruz), NCAM (Mouse anti-mouse/rat, 1:1000, Hybrdoma Bank), and NFASC155 (Rabbit antimouse, 1:1000, Abcam). The blots were washed and incubated for 1 h at room temperature (RT) with AP-conjugated secondary antibody (1:1000; Dako, Glostrup, Denmark), or HRP-conjugated secondary antibody (GE Healthcare, Diegem, Belgium, 1:10,000). Immunodetection was performed using the ECF immunoblotting detection system for AP-conjugated secondary antibody (GE Healthcare, Diegem, Belgium), or Super Signal West Femto for HRP-conjugated secondary antibody (Thermo scientific, Rockford, USA). Blots were scanned with the FLA-5000 (Fuji Photo Film Corp.), or with the Li-cor Odyssey system, respectively. Relative amounts of immunoreactivity were quantified using Quantity One software (Bio-Rad). To correct for input differences, either Coomassie staining (the upper or lower half of the same gel for ECF), or the stain-free (TCE containing gel for Femto) activated signal was used.

**Myelin isolation and immunoblotting of purified myelin**

Myelin was isolated from whole brain extracts as described by Menon et al. [51]. In short, brains were homogenized in 8.5 mL solution of 0.32 M sucrose, 2 mM EGTA (pH 7.5) and complete EDTAfree, protease inhibitory cocktail (1 tablet/50 mL of homogenization buffer; Roche Applied Sciences, Indianapolis, IN, USA). Next, sucrose gradient isolation steps were performed using first 0.85 M, and subsequently 1 M sucrose solutions, alternated with sugar wash out (in 10 mM EGTA in nanopure water, pH 7.5) and osmotic shock (in 10 mM EGTA in nH2O, pH 7.5). Myelin fractions were suspended in 50 mM Tris (pH 7.5) with protease inhibitor cocktail, and analyzed using immunoblotting as described above. Membranes were probed with primary antibodies as described above. In addition, L-MAG was probed with an additional L-MAG Rabbit anti-mouse antibody, 1:500 (a kind gift from Prof. N. Schaeren-Wiemers, University Hospital Basel, Basel, Switzerland [52]).
Chapter 4: Genetic Variation in Myelination of RI Mice

**Conduction velocity measurements**

C57 (n=4) and DBA (n=5), 2 months old, were decapitated and the brains were rapidly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF; containing NaCl 129 mM, KCl 3 mM, MgSO4 1.8 mM, CaCl2 1.6 mM, glucose 10 mM, NaH2PO4 1.25 mM, NaHCO3 21 mM w. pH7.4); carboxygenated with 5% CO2 and 95% O2. Coronal slices (400 μm) were acutely prepared from the frontal cortex including corpus callosum. After sectioning, slices were maintained and recorded at RT(20–22°C) in ACSF. Extracellular field currents were recorded with Heka EPC-8 amplifiers (D-67466 Lambrechtt/Pfalz, Germany). The ACSF-filled glass microelectrodes were voltage clamped at 0 mV. The measurements, performed on slices of C57 mice (n=14) and DBA mice (n=11), were taken from three different locations along the corpus callosum with platinum/iridium electrodes (FHC, Bowdoin, ME 04287, USA). Data were low-pass filtered at 5 kHz, digitized at 20 kHz, with an instrutech ITC-16 and pulse software (D-67466 Lambrecht/Pfalz, Germany) and analyzed off-line with Igor Pro (Wavemetrics, 10200 SW Nimbus, G-7, Portland, USA). Evoked currents were measured using two different recording electrodes and were abolished by tetrodotoxine (TTX), a selective blocker of voltage-gated sodium channels.

**Electron microscopy (EM) and morphometric analysis**

Animals under deep anesthesia were perfused transcardially with 20 mL of PBS 0.1 M (pH 7.4) containing 0.1% heparin, followed by 100 mL of freshly prepared cold fixative solution composed of 4% paraformaldehyde in 0.1 M PBS and 2% glutaraldehyde (pH 7.4). Optic nerves were removed, postfixed overnight in fixative solution at 4°C, and cryoprotected with 30% sucrose for 2–3 days at 4°C. Optic Nerves were rapidly frozen in powdered dry ice and sliced into ultra-thin cross sections on a cryostat (–20 °C).

Free-floating sections were rinsed three times, dehydrated with ethanol and embedded in Epon. Ultra-thin sections were subsequently cut, collected on formvar-
coated single slot grids, and stained with a 1% aqueous uranyl acetate solution for 20 minutes and subsequently for 1 minute with lead citrate. Photographs were obtained by using a JEOL 1010 electron microscope. For each myelinated axon present the g-ratio was calculated by dividing the axonal diameter (defined by the inner limit of the myelin sheath) by the total fiber diameter (defined by the outer limit of the myelin sheath). Each group consisted of at least 3 mice at P60, and 120–140 axons per mouse line were counted.

Behavioral measurements and correlations with myelin expression data from hippocampus

Behavioral testing was performed by using the dark light box, open field, 5-choice serial reaction time task, and pre-pulse inhibition test (PPI), and described by us previously [43, 45, 46], except for the dark light box. Mice were introduced into the dark compartment (<10 lux, length x width x height: 25 x 25 x 30 cm) of a dark light box. Then, 60 s later a door opened providing access to an identical sized compartment that was brightly lit (325 lux) and left open for 10 minutes. Visits to, and time spent in the light compartment were counted when the body reference point of a mouse protruded at least 2 cm into the light compartment away from the door, as assessed by video tracking (Ethovision 3.1, Noldus Information Technology, Wageningen, The Netherlands). Outlier analysis was performed using 2*SDEV as exclusion criteria. Pearson correlations between the mean myelin gene expression value per strain as derived from Hippocampus Consortium M430v2 Jun06 data set, and the mean behavioral data per strain, were performed on either raw data, or Log10 transformed data if not following a normal distribution. Analyses were performed on BXD strains (n=36 to n=40, depending on the test), with n>10 individuals per strain.
Results

*Myelin-associated gene expression shows large differences between C57, DBA and their BXD progeny*

To determine possible myelin-related gene expression differences between C57, DBA and their BXD offspring, expression data was extracted from the GeneNetwork database using the high quality brain dataset Hippocampus Consortium (Build Jun06) [28]. Various transcripts were investigated that are known to be associated with different myelin compartments, e.g., compact myelin (PLP, MBP, MAL, MOBP), non-compact myelin (MAG, MOG), and radial component (Claudin11). Clustering of the myelin transcript data over strains was performed as described in the methods section. This analysis identified strong differences with a cluster pattern that was very consistent over all the myelin associated genes selected, i.e. if a BXD strain showed a higher or lower expression of a myelin gene compared with the median of this gene for all BXD strains combined, this would in general be seen for all other myelin genes as well (figure 1A). For other functional groups, this co-regulation pattern of genes over all BXD strains was not found, i.e. gene expression of axonal/dendritic markers (figure 1B), and lipid metabolism (figure 1C). The difference in the mean of the myelin gene group expression between strains was a maximal 3.3-fold. Both the parental strains, C57 and DBA, were on the opposite extreme ends of the myelin gene expression spectrum. This analysis was successfully replicated in the smaller INIA Brain mRNA database (Build Jan06; figure 2).
Chapter 4: Genetic Variation in Myelination of RI Mice

A Myelin gene expression

Fold Change = 3.30

B Axonal/dendritic gene expression

C Lipid gene expression
Figure 1. Clustering of gene expression data shows pronounced differences in myelin gene expression between C57, DBA, and their BXD progeny. BXD microarray data was obtained from the GeneNetwork ‘Hippocampus Consortium M430v2 Build Jun06 PDNN’ dataset. Transcripts were selected and clustered with TMEV software, using Euclidean distance and average linkage clustering. A) The clustering pattern indicates a clear group regulation of all myelin transcripts investigated, with the parental strains C57 and DBA opposite at the most extreme ends. B), C) Clustering of transcript data related to axonal/dendritic markers and lipid metabolism, respectively, did not reveal similar patterns of co-regulation. Number of strains is 71. Scale bar is depicting Log2 values. The intensity threshold was set to 0 for all depicted functional groups (i.e. myelin, axonal/dendritic and lipid genes).

Figure 2. Clustering of gene expression from the INIA Brain dataset shows pronounced differences in myelin gene expression between C57, DBA, and their BXD progeny. BXD microarray data was derived from the GeneNetwork ‘INIA Brain mRNA M430 Build Jan06 PDNN’ dataset. A) Myelin transcripts were selected and clustered with TMEV software, using Euclidean distance and average linkage clustering. A clear clustering pattern involving all myelin transcripts was detected, with the parental strains C57 and DBA at the most extreme ends. Number of strains is 41. Scale bar is depicting Log2 values. The intensity threshold was set to 0. B) Correlation with the independent Hippocampus Consortium M430v2 Jun06, PDNN database. r = 0.43, p<0.01. Number of strains is 37.

qPCR analysis independently confirms significant differences in myelin gene expression between C57 and DBA mice

To validate the microarray-based data from GeneNetwork (Build Jun06 and Jan06), qPCR analysis was performed both on hippocampi and on tissue from the rest of the brain derived from parental strains C57 and DBA, with ages 10 to 12 weeks. The hippocampi of C57 mice showed 3 to 5-fold higher levels of myelin transcript levels than DBA (figure 3A). Similar higher levels for myelin transcripts were found in total
brain (brain minus hippocampus), albeit to a lesser extent (figure 3B). Together these data confirm that C57 and DBA are different in the expression of myelin-associated genes.

![Graphs of myelin gene expression](image)

**Figure 3. qPCR analysis confirms myelin gene expression differences between the parental C57 and DBA strains.** A) qPCR was performed on myelin gene transcripts in hippocampi from C57 and DBA mice. For nearly all myelin genes investigated, differences were significant, with C57 mice showing higher levels of expression. B) qPCR analysis on myelin gene transcript in whole brain tissue (minus hippocampus). DBA, n = 5; C57, n = 6. T-test (1-tailed) **p<0.01; *p<0.05.

**Protein analysis reveals differences in myelin protein expression between C57 and DBA strains and indicates specific differences in composition of the myelin sheath**

To check whether the genetic differences in myelin transcript levels also extent to protein levels, immunoblotting from whole brain homogenates (including hippocampus) was performed on several proteins associated to the different myelin compartments (figure 4). To determine potential developmental effects, the analysis was performed on P20 (pups) and P62 (adult) mice. Myelin protein MOBP was found to be higher expressed in C57 in both pups and adults, whereas CNP and Pan-MAG (i.e. P-MAG, recognizing both L- and S-MAG isoforms; thus total MAG) were higher.
Chapter 4: Genetic Variation in Myelination of RI Mice

A. Myelin protein expression in whole brain extracts of P20 pups

B. Myelin protein expression in whole brain extracts of adult mice

Figure 4: continued on next page
Figure 4. Differences in myelin protein expression between C57 and DBA mice in whole brain extracts. Immunoblotting was performed for selected myelin proteins on total brain extracts of P20 (A, C) and P62, adult mice (B, D). A, B) Graphs showing the quantification of myelin proteins measured in pups (P20) and adult (P62) mice, respectively. C, D) Blots of quantified myelin proteins shown in A and B for P20 pups and P62 adult mice, respectively. Coomassie gel staining depicted is a representative example blot, taken from one of the quantified myelin proteins shown in A and B. Significant differences represented by: T-test (2-tailed) **p<0.01; *p<0.05. #, Similar results were found for all measured MBP isoforms shown in C, D. L-MAG: Large MAG isoform; P-MAG: Pan-MAG, recognizing large and small MAG isoforms, respectively. N = 3–4 mice per group.
expressed in C57 adults only. Remarkably, NCAM-120 (the predominant isoform of NCAM expressed by oligodendrocytes [53-55]) showed lower expression in C57 pups, and L-MAG (i.e., large isoform) showed lower expression in both C57 pups and adults. Overall, the myelin protein expression thus followed its mRNA levels, with higher expression in C57 mice, with the exception of NCAM-120 and L-MAG.

To determine whether C57 and DBA were also different in protein composition of the myelin membrane, immunoblotting was performed on purified myelin samples from whole brain extracts. Indeed, myelin of C57 mice contained higher levels of MOBP and CNP, whereas DBA contained higher levels of L-MAG and NCAM-120 (figure 5). We conclude that C57 and DBA brains are strongly different in expression of myelin proteins and in myelin protein composition.

Figure 5: legend on next page
Figure 5. Differences in myelin protein expression between C57 and DBA mice in purified myelin

**Samples.** Immunoblotting was performed for selected myelin proteins on purified myelin derived from C57 and DBA brains, at P20 (for CNP and MOBP) or adult (NCAM-120 and L-MAG). A) Graphs showing quantification of all proteins measured. B) Blots for the myelin proteins. The depicted Coomassie gel staining is a representative example blot, taken from one of the quantified myelin proteins shown in A. Significant differences represented by: T-test (2-tailed) **p<0.01; *p<0.05. L-MAG: Large MAG isoform, measured and validated with 2 different antibodies; e.g. L-MAG 1 (Santa Cruz; that was also used for measurements on the whole brain homogenates) and L-MAG 2 (a kind gift from Prof. N. Schaeren-Wiemers, University Hospital Basel, Basel, Switzerland). N = 3–4 mice per group.

*Electron microscopy (EM) reveals smaller myelinated fibers in DBA mice*

To further investigate myelin protein differences between C57 and DBA at 2 months of age, EM analysis was performed on optic nerves: a CNS structure in which all axons are myelinated, making it possible to determine small differences in myelination (figure 6A, B). No differences in g-ratio were found between C57 and DBA (figure 6C, D). We found that myelin thickness (figure 6E) was smaller in DBA compared with C57. This is of interest in light of higher levels of myelin transcripts (figures 1-3) observed in C57, and higher levels of a subset of myelin proteins (figures 4-5). Based on a similar g-ratio and smaller myelin thickness in DBA, this would imply that DBA mice have smaller diameter axons. We indeed confirmed that myelinated fibers in the optic nerve are smaller in DBA compared with C57 mice (figure 6F).
**Figure 6. Smaller myelinated fibers in optic nerves of DBA mice.** A, B) EM analysis of optic nerves in cross-sections of either C57 or DBA, respectively, in mice at 2 months of age. C, D) Morphometric g-ratio analysis of myelinated axons in optic nerves of C57 and DBA. E, F) Morphometric analysis of general myelin thickness and axon diameter of myelinated axons in optic nerves of C57 and DBA. Scale bar, 2 μm. T-test (2-tailed) *p<0.05.

*Higher axonal conduction velocities in myelinated axons of C57 mice*

To determine whether the observed differences in myelin protein composition, and in myelinated fiber size, caused differences in the velocity of action potential propagation in the brains of C57 versus DBA mice, we measured conduction velocity in the corpus callosum of C57 (n=5) and DBA (n=4) mice at 2 months of age. Higher conduction velocities were found for the fast conducting fibers in the corpus callosum of C57 mice, when compared with DBA mice (figure 7). Because fast conducting fibers are myelinated, in contrast to the slower conducting fibers, this indicates that the differences observed in myelination and myelin composition contribute to differences in functional connectivity in the brains of C57 and DBA mice.

**Figure 7. C57 mice show, in comparison with DBA, increased signal conduction velocity for fast, myelinated fibers in the corpus callosum.** A) Typical example of a conduction velocity measurement. A fast signal is detected, corresponding to fast conducting myelinated fibers, and a slow signal, corresponding to slow conducting nonmyelinated fibers. B) A significant difference in conduction velocity was observed for the fast signal. Significant differences represented by: T-Test (2-tailed) **p<0.01. C57, N=4 (14 slices); DBA, N=5 (11 slices).
**Hippocampal myelin gene expression correlates with behavioral traits in C57, DBA and their BXD progeny**

Next, we investigated whether the differences in myelin gene expression, and white matter conduction velocity, might relate to behavioral differences in various domains observed between C57, DBA and their BXD offspring, as previously assessed by us [43, 45, 46]; except for the dark light box for which results have not been previously published. Significant, positive correlations were found between myelin gene expression in the hippocampus and specific behavioral measures of activity and/or anxiety in open field and dark light box (table 2). This indicates that higher myelin transcript levels might be related to higher activity levels and/or less anxiety as measured by moving around longer and more frequently in open and light spaces and compartments. Correlations were not significant for measures of impulsivity and attention as determined by the 5-choice serial reaction time task, and for measures of sensorimotor gating in a pre-pulse inhibition test (PPI). Taken together, we conclude that myelin gene expression levels correlate with activity and/or anxiety levels of BXD mice, indicative of a role for genetic variance in myelination in brain functioning and behavior.

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Significant correlations are shown in **bold** and *cursive*. **p<0.01; *p<0.05. Abbreviations: r = Pearson product-moment correlation coefficient, N = number of BXD strains, including parental C57 and DBA. N of mice per strain for gene expression values varies between 2 and 4, for behavioral analysis >10.

**Discussion**

The BXD panel of mouse RI strains is a GRP, or genetic reference population, for modeling of genetic complexity, such as present in the human population [19]. The parental strains, C57 and DBA, are known to differ in many behavioral, molecular and functional correlates. Here, we set out to investigate whether genetic differences in myelin transcription are present in BXD lines, which may underlie brain molecular, functional and behavioral differences in these strains.

We found robust differences in expression of myelin transcripts in a BXD panel, for all myelin genes investigated. These results are in line with literature on myelin gene expression, indicating presence of transcription factors that specifically regulate expression of groups of oligodendrocyte and myelin genes [56-61]. The parental C57 and DBA strains were on the opposite ends of the expression spectrum, with higher myelin gene expression in C57. This is in concurrence with a previous microarray study, in which the expression of a cluster of myelin and myelin-related genes in prefrontal cortex (PFC) was reported higher in C57 mice compared with DBA mice [62]. Interestingly, in this study it was shown that myelin genes were differentially responsive to acute ethanol treatment in PFC from C57 versus DBA, indicating that myelin genes might play a dynamic role in effects of ethanol on white matter tracts; and hence on inter-neuronal signaling in the brain [62]. Together with our observation that myelin gene and protein expression differences between C57 and DBA are not exclusively present in the PFC, but also apparent for other white matter containing regions (such as corpus callosum, hippocampus, and optic nerve) under naïve conditions, this leads to the question whether genetic variation in myelin gene expression might also contribute to previously reported differences in signaling and plasticity processes in C57 and DBA mice [36-41].
Next, we focused on the parental C57 and DBA strains to investigate whether the higher expression of myelin gene transcripts in C57 would translate to differences on the protein level. To take into account possible developmental effects, analyses were performed on adult animals, and on animals age P20, as around this age substantial postnatal myelination takes place in brains of mice. In both adults and pups, variation in myelin expression between C57 and DBA was only reflected for a small subset of myelin proteins. This indicates that the lower expression of myelin gene transcripts in DBA does not slow down the general onset and development of myelination in these mice compared with C57, as this would likely involve lower expression of all investigated major myelin proteins during peaks of myelination.

The fact that not all myelin gene expression differences found on transcript level were reflected at the protein level, is not uncommon considering the many regulatory processes that can occur in-between gene transcription and protein expression [63]. Nevertheless, we found that the differences in transcript levels did translate to higher protein expression in C57 for MOBP, CNP and total MAG, the latter in adults only. Remarkably, L-MAG isoform and NCAM-120, the latter in pups only, showed a lower expression in C57 compared with DBA mice. These results in whole brain homogenates could be validated in purified myelin samples. Taken together, the myelin protein expression differences mostly followed the mRNA levels, with higher expression in C57 mice, though isoform protein expression of L-MAG and myelin-associated NCAM-120 showed an opposite pattern. The much lower expression of L-MAG in C57, together with similar to higher levels of total MAG in C57, suggests lower levels of S-MAG in DBA. It could thus be hypothesized that the higher expression of L-MAG isoform in DBA might represent a compensatory mechanism. Interestingly, NCAM has been suggested to be an adhesion protein similar in structure to MAG, that might also help compensate for the absence of MAG in MAG-null mice during myelin formation [64], or that might partially substitute for MAG in the maintenance of axon-myelin integrity [65].
Despite these potentially compensatory mechanisms in DBA mice with respect to L-MAG and NCAM-120 protein isoforms, conduction velocity measurements showed specifically reduced action potential propagation for the fast, myelinated fibers in the corpus callosum of DBA compared with C57. This indicates that the differences in myelin gene expression and protein composition might underlie functional differences in brain connectivity and signaling.

Although we did not find differences in g-ratio of myelinated fibers in optic nerves of C57 and DBA, myelinated fibers of DBA had smaller axon diameters and thinner myelin. Both thickness of the myelin sheath and the diameter of axons is a well-known feature that influences the speed of signal transduction in axons. Interestingly, studies in both MAG- and MOBP-deficient mice have indicated that these glial proteins might influence axonal diameter [66-68]. MAG knockout mice have smaller myelinated fibers in both the CNS and PNS, with thinner myelin and smaller diameter axons. Furthermore, nerves in the PNS of these mice were shown to have reduced conduction velocities [68-70]. Strikingly, our observation of smaller myelinated axons in DBA, together with higher L-MAG levels, is in line with reported differences for expression of L-MAG and S-MAG isoforms on small and large-diameter axons respectively; L-MAG is primarily associated with smaller oligodendrocytes that myelinate numerous relatively small axons (also known in classical nomenclature as type I and II oligodendrocytes), whereas the highest levels of S-MAG are found in the larger type III and IV oligodendrocytes, that myelinate only a few larger axons [66, 71]. MAG has been implicated in signaling in both directions between glia and axons. Axonal signaling might cause activation of cyclin dependent kinase 5 (Cdk5) and extracellular signal regulated kinases 1 and 2 (Erk1/2) and increased expression of phosphorylated neurofilaments leading to larger axonal caliber and probably other axonal changes needed for maintenance and survival [66]. Signaling in the opposite direction, enhances the capacity of oligodendrocytes to form and maintain myelin [66]. With the L-MAG isoform being particularly important for signaling in
oligodendrocytes, and the S-isoform for the axonal signaling [66], this might explain the smaller diameter axons observed in the DBA mice.

More subtle differences in myelin composition and structure might have an influence on conduction velocity and signaling in the brains of C57 and DBA as well. Myelin abnormalities have been observed using regular EM techniques in MAG, MOBP and CNP mutant mice, and include redundant and supernumerary myelin sheaths, and irregularities in the periaxonal, inner tongue or paranodal regions of myelin sheaths and axons [53, 64, 66-70, 72-77]. These ultrastructural abnormalities were however not the subject of our EM quantitative analysis that focused on g-ratio and myelin thickness. Further studies might elucidate whether the differential expression of myelin transcripts and proteins in C57 and DBA are associated with more subtle structural changes in myelin and myelinated axons as well.

Interestingly, we also found that the BXD myelin gene expression data correlated with differences in anxiety/activity behavioral traits. Several studies have emerged that point at conduction velocity and processing speed as predictors of general intelligence [5, 6], or that report correlations between processing speed and cognitive impairments for patients with multiple sclerosis [78]. We hypothesize that our myelin-related findings may (partly) underlie behavioral differences between C57 and DBA mice. Interestingly, the correlations were only found for anxiety/activity behavioral traits, and not for measures of impulsivity or pre-pulse inhibition (PPI). This indicates that the here reported genetic differences in myelination might influence specific behaviors only. Nevertheless, further validation is needed to gain more insights in robustness of the here reported correlations. Due to the huge amount of available phenotypic data for the BXD panel in public databases as GeneNetwork, validation of our findings is compromised by the high probability of false positive significant correlations. An overrepresentation analysis as described in Pietrzykowski et. al., [29], will help to tackle this issue.
Noteworthy, the role of myelin in signaling in the brain is not solely a matter of regulating the speed of compound action potentials; recently, it has become clear that myelin also plays an important role in the support of long-term integrity of axons [79]. In line with this, MAG, MOBP and CNP-deficient mice all have defects in the maintenance and support of myelinated axons, including differences in neurofilament spacing, focal swellings and spheroids, axonal loss and in some cases neurodegenerative symptoms [53, 64, 66-69, 72-77, 79]. In addition, it is known already for some years that MAG as an inhibitory myelin protein, can also influence neurite outgrowth, and thereby affect the formation of synaptic connections and circuits during development and regeneration [66]. Such influences would not necessarily be measurable by conduction velocity measurements in myelinated axons, however, these might have prominent effects on brain wiring, and ultimately cognition and behavior as well. Interestingly, DBA mice are used as a well-known animal model for studies of age-related glaucoma, with distal axonal injuries in optic nerve appearing as an early symptom [80]. In addition, DBA mice are more vulnerable than C57 for the development of neurodegeneration induced by systemic administration of kainic acid (KA) [81], and for age-related neurodegeneration in the PNS, showing more lesions at an earlier age [82]. Moreover, general differences in synapse and neuronal circuitry formation between C57 and DBA are well appreciated and studied [83-86]. It remains to be determined whether the differential expression of myelin proteins in C57 and DBA may contribute to these features.

In conclusion, we report significant differences in myelin between C57 and DBA on the molecular level (myelin transcript and protein) and the structural level (smaller, myelinated axons in DBA). We propose that these differences might underlie those found for conduction velocity and specific behavioral phenotypes, such as activity and anxiety.
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