Antagonistic Functions of MBP and CNP Establish Cytosolic Channels in CNS Myelin

Highlights
- Characterization of “cytoplasmic channels” in myelin close to their native state
- Antagonistic functions of MBP and CNP in generating cytosplasmic channels
- CNP interacts with and bundles actin
- Reducing MBP levels rescues axonal pathology in CNP-deficient mice

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In Brief
Snaidero et al. provide evidence that a system of cytoplasmic-rich channels is generated in myelin sheaths by the antagonist function of MBP and CNP. The authors suggest that these channels are required to provide trophic support to neurons and maintain functional axon-glial units over a long period of time.
AntAGONISTIC FUNCTIONS OF MBP AND CNP
EStABLISH CYTOSOLIC CHANNELS IN CNS MYELIN

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SUMMARY

The myelin sheath is a multilamellar plasma membrane extension of highly specialized glial cells laid down in regularly spaced segments along axons. Recent studies indicate that myelin is metabolically active and capable of communicating with the underlying axon. To be functionally connected to the neuron, oligodendrocytes maintain non-compacted myelin as cytoplasmic nanochannels. Here, we used high-pressure freezing for electron microscopy to study these cytoplasmic regions within myelin close to their native state. We identified 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), an oligodendrocyte-specific protein previously implicated in the maintenance of axonal integrity, as an essential factor in generating and maintaining cytoplasm within the myelin compartment. We provide evidence that CNP directly associates with and organizes the actin cytoskeleton, thereby providing an intracellular strut that counteracts membrane compaction by myelin basic protein (MBP). Our study provides a molecular and structural framework for understanding how myelin maintains its cytoplasm to function as an active axon-glial unit.

INTRODUCTION

In the CNS, myelin is formed by oligodendrocytes that spirally wrap their plasma membrane around axons. Previously, myelin has been regarded as an inert and purely insulating membrane, but it is now clear that myelin is metabolically active, providing support to the underlying axon (Fünschilling et al., 2012; Lee et al., 2012; Saab et al., 2016). In addition, myelin growth in response to neuronal activity has been described, and this may contribute to information processing by modulating velocity and synchronicity of nerve impulses in neuronal networks (Fields, 2015; Chang et al., 2016). At first glance, structural dynamics seems to be incompatible with myelin consisting of multilamellar membrane with little cytoplasm (Snaidero and Simons, 2014). However, most of what we know about myelin ultrastructure is based on electron microscopic studies performed on chemically fixed and dehydrated tissue, often associated with shrinkage and collapse of intracellular spaces.

A recent technical advance has been the application of high-pressure freezing electron microscopy to biological tissues leading to an enhanced preservation of tissue and cell architecture, including the cytoplasmic spaces within myelin (Möbius et al., 2010; Weil et al., 2016). With this technique, it is possible to visualize within the developing myelin sheath a system of tube-shaped cytoplasmic expansions residing between the compacted layers of myelin (Snaidero et al., 2014). These channels run through the compacted sheath, connecting the oligodendroglial cell body, the major site of membrane biosynthesis, to the innermost layer of myelin, which is in direct contact with the axon. These cytoplasmic regions are reminiscent of Schmidt-Lanterman incisures (cytoplasmic incisures of peripheral nervous system myelin) and also comprise the paranodal loops and the outer and inner periaxonal “tongues” of myelin. The detection of microtubules and vesicular structures within the cytoplasmic regions suggests that they serve as tracks for motor-driven transport processes. To what extent these cytoplasmic channels persist in adult myelin after completed myelination is not known.

Membrane compaction closes most of the cytoplasmic regions in myelin and is mediated by myelin basic proteins (MBPs), the major structural component of myelin. MBP is an intrinsically disordered polypeptide chain with a strong basic character, which is able to bind to the two apposing negatively charged cytoplasmic leaflets of the myelin membrane (Harauz et al., 2009). This interaction neutralizes the positive charge in
MBP and triggers self-assembly into a polymeric network (Aggarwal et al., 2013). Polymerization of MBP molecules onto and between membranes provides the means to extrude cytoplasm from the myelin sheath (Aggarwal et al., 2011).

Given the function of myelin in supporting axonal integrity, we now asked how cytoplasmic channels are formed and maintained in the developing and adult nervous system. We identified 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), an oligodendrocyte-specific protein previously implicated in the maintenance of axonal integrity (Lappe-Siefke et al., 2003), as an essential factor in the maintenance of intact cytoplasmic regions in the adult myelin sheath. We provide evidence that CNP antagonizes the activity of MBP in compacting myelin membrane layers. We propose that CNP counteracts membrane zippering by associating with and organizing the actin cytoskeleton within the cytoplasmic regions of the myelin sheath, thereby keeping the adjacent cytoplasmic leaflets separated and preventing excessive membrane compaction by MBP.

RESULTS

CNP and MBP Determine the Amount of Cytoplasm within Myelin Sheaths

To analyze the role of CNP and MBP in the biogenesis of cytoplasmic channels in the developing myelin sheath, we determined the number of these channels in the optic nerve in mice lacking CNP (CNP-deficient) or a decreased dosage of MBP (heterozygous shiverer) at postnatal day 10 (P10), P14, and P21 (Figures 1A–1C). We used high-pressure freezing and freeze substitution for electron microscopy to visualize the cytoplasmic regions within the myelin sheath of the developing optic nerve. As shown previously, we find that a large fraction of the cytoplasmic regions disappears with the maturation of the myelin sheath (Snaidero et al., 2014). Strikingly, when CNP-deficient animals were analyzed, we observed a decrease of cytoplasmic spaces in myelin. In CNP-deficient mice, the number of cytoplasmic pockets visualized by electron microscopy in cross sections was reduced by ~40%, ~70%, and ~80% compared to wild-type controls at P10, P14, and P21, respectively. In contrast, when heterozygous shiverer (Mbp<sup>+/−</sup>) mice, which are well myelinated, were analyzed and compared to wild-type animals, we observed a transient increase in the number of cytoplasmic regions when compared at P14 (Figure 1C). This is reminiscent of increased numbers of Schmidt-Lanterman incisures in the PNS of heterozygous shiverer (Mbp<sup>+/−</sup>) mice (Gould et al., 1995).

Since the cytoplasmic regions are sparse in thin-caliber axons of the adult optic nerve, we analyzed the spinal cord, which contains thicker myelin sheaths with more cytoplasm (Blakemore, 1969). To study the structure of myelin of large-caliber axons, we optimized high-pressure freezing for spinal cord tissue. We found that 5 min of pre-fixation (with paraformaldehyde/glutaraldehyde) of the spinal cord followed by embedding in gelatin and the subsequent cutting of 200-μm-thin sections greatly enhances tissue quality for high-pressure freezing. Using this protocol, we find that cytoplasmic regions are more frequent in large-caliber axons with thick myelin sheaths (Figures 1D–1K). When analyzing an earlier time point (P15), more cytoplasmic regions were found in thick myelin sheaths of the spinal cord as compared to P60 and P180 (Figure S1). However, contrary to the optic nerve, a large fraction of these cytoplasmic regions within the thick myelin sheaths remained into adulthood (Figures 1D–1K). When CNP-deficient mice were analyzed and compared to wild-type mice, we observed a striking reduction in the number of cytoplasmic regions within myelin (>300 nm thickness) both at P60 (Figures 1D and 1F) and P180 (Figures 1E–1G). In heterozygous shiverer mice (Mbp<sup>+/−</sup>) cytoplasmic regions in myelin sheaths (>600 nm thickness) were significantly increased. However, contrary to the CNP-deficient mice, in which these abnormalities persist, heterozygous shiverer mice were not significantly different from wild-type mice at P180, showing that the effects of MBP on the cytoplasmic channels are transient (Figures 1H–1K).

Antagonistic Function of CNP and MBP in Membrane Compaction

MBP is the prototype compact myelin protein, whereas CNP is thought to be enriched in non-compacted regions. We performed immunoelectron microscopy and observed that CNP is indeed highly enriched in the cytoplasmic regions of myelin and almost excluded from compacted myelin (Figure S1). How does CNP determine the number of cytoplasmic channels within myelin?

Our results point to an antagonistic function of CNP and MBP in maintaining cytoplasm within myelin sheaths. One possibility of how CNP could exert such a function is by forming pillars in the cytoplasmic regions of the myelin sheath. Such pillars may keep the adjacent cytoplasmic leaflets separated, thereby preventing membrane compaction by MBP. To test this idea, we used our recently established biomimetic in vitro compaction assay (Aggarwal et al., 2013), which examines the interaction of giant unilamellar vesicles (GUVs) with supported lipid bilayers (SLBs) coated with MBP. In this system, MBP is sandwiched between a SLB and GUVs, and its adhesive and self-interacting properties induce the spreading of GUVs onto the SLB (see graphical illustration of the assay in Figure 2). First, we determined the critical concentration of MBP required for GUV spreading. Different concentrations of recombinant MBP (14-kDa isoform) were added onto the SLBs before fluorescently labeled GUVs were placed on top of MBP-decorated SLBs. We found that 0.4 μM MBP was necessary to initiate the bursting of the GUVs onto the SLBs (Figure S2). We next tested whether recombinant CNP could prevent the spreading of GUVs onto the SLBs induced by MBP. Since CNP is a lipid-anchored membrane protein, we designed a recombinant variant of CNP with a small stretch of positively charged amino acids at its C terminus to link it to the negatively charged SLBs. GFP containing the same tag for membrane binding was used as a control. We found that CNP, but not GFP, was able to antagonize MBP-mediated spreading of the GUVs onto the SLBs. Thus, using a simplified in vitro compaction assay, we have reconstructed the antagonistic role of CNP and MBP (Figure 2).

CNP Counteracts Membrane Compaction by Associating with and Organizing the Actin Cytoskeleton

Previous studies have shown that CNP co-immunoprecipitates with actin from cell lysates (De Angelis and Braun, 1996), but
whether CNP interacts directly with filamentous actin (F-actin) and the relevance of such an interaction for myelin compaction is not known. We used recombinant CNP variants (including full-length CNP, the N-terminal domain alone, the C-terminal catalytic domain alone, a variant of the catalytic domain extending all the way to the C terminus, and an inactive mutant of the catalytic domain) and actin purified from muscle to characterize direct protein-protein interactions in vitro.

We carried out in vitro F-actin co-sedimentation assays with high-speed ultracentrifugation and found that CNP pelleted with microfilaments, behaving like a typical F-actin-binding protein (Figures 3A and S3). By titrating CNP, we observed that the...
The maximal amount of co-sedimented full-length CNP was close to the amount of actin in the pellet, suggesting a 1:1 stoichiometry for binding (Figure S3A). We also tested the N- and C-terminal domains of CNP and found that they both bound to F-actin independently (Figures 3A and 3B). Adding the C-terminal 22 residues to the catalytic domain (Figure 3A), which are believed to be important for membrane anchoring and microtubule interactions, did not affect F-actin co-sedimentation. Furthermore, an enzymatically inactive mutant of the catalytic domain, in which both active-site His residues are replaced by Gln, also similarly co-sedimented with F-actin, showing that CNPase activity is not required for the interaction (Figure S2B). The CNP interaction partner calmodulin (CaM) (Myllykoski et al., 2012) prevented CNP co-sedimentation into F-actin pellets, while another EF-hand protein abundant in myelinating glia, S100β, did not (Figures S3C and S3D). Thus, molecular interactions with competing partners may regulate the CNP-actin complex. Furthermore, a small increase in the actin polymerization rate was observed with CNP (Figure S3E).

Next, we used low-speed centrifugation to analyze the F-actin bundling activity of full-length CNP and its catalytic domain. Both constructs showed clear bundling activity, and the effect was already seen at 1 μM CNP (Figure 3D). The same effect was observed for the catalytically inactive mutant. We further performed electron microscopy on CNP-bundled microfilaments. Immunogold labeling showed F-actin bundles decorated with full-length CNP, while areas devoid of F-actin contained no CNP (Figure 3C).

To further map the interaction stoichiometry and potential binding surfaces, we carried out covalent crosslinking of CNP and F-actin, followed by mass spectrometric peptide mapping. When both CNP and actin were present in the crosslinked sample, specific patterns of bands were observed on SDS-PAGE, indicating protein-protein complex formation. In addition to a 1:1 species, also higher oligomeric states were resolved (Figure 3E). The oligomerization pattern was similar between full-length CNP and the catalytic domain, indicating that the C-terminal domain is sufficient to drive an interaction between CNP and F-actin (Figure 3E). To determine the interaction sites, several bands from electrophoresis were processed for tryptic peptide mapping. All picked crosslinked hybrid bands contained both actin and CNP as shown by matrix-assisted laser desorption-ionization time of flight (MALDI-TOF). The peptide pattern determined by mass spectrometry was used to predict the interaction sites. For actin, the binding appears to occur near the D-loop in subdomain 2 as well as the long loop of subdomain 3 and for CNP on the surface of the N-terminal PNK-like domain (Figure 3F).

Taken together, the results from co-sedimentation and crosslinking demonstrate that CNP can bind microfilaments directly and induce their bundling. Both domains of CNP bind F-actin, and the observed effects are independent of CNP catalytic activity.

Having demonstrated that CNP is able to bind and bundle F-actin, we used our in vitro compaction assay to determine the effect of F-actin on MBP-mediated membrane spreading. We found that F-actin by itself was not able to block MBP-mediated spreading of GUVs onto SLBs (Figure 3G). However, when F-actin was added to SLBs, which had been pre-coated with CNP, MBP-mediated spreading of the GUVs was fully blocked.

Figure 2. Antagonistic Function of CNP and MBP in Membrane Compaction

(A and B) Biomimetic membrane system to reconstitute the function of MBP in vitro. MBP is sandwiched between supported lipid bilayers (SLBs; mimicking the inner leaflet composition of myelin) and giant unilamellar vesicles (GUVs). (A) SLBs were coated with 7 μM membrane-anchored GFP (R3-GFP), followed by the addition of purified recombinant 14-kDa MBP (0.4 μM), on top of which GUVs composed of PS and PC in 1:2 molar ratios (0.1 mol% of DHPE-Texas red was used to visualize the GUVs) were added. After 30 min, all the GUVs burst on the SLBs (burst GUVs appear as red fluorescent areas). (B) SLBs were coated with 7 μM membrane-anchored CNP (R3-CNP) followed by the addition of purified recombinant 14-kDa MBP (0.4 μM), on top of which GUVs were added. After 30 min, the R3-CNP partially prevented the bursting of the GUVs (non-burst GUVs appear as dark spheres with fluorescent rim). Bursted GUVs are marked by a white arrowhead, whereas unbursted GUVs are marked by a black arrowhead. Scale bar, 10 μm.

(C) Quantification of GUV bursting over time.

(D) Model illustrating the biomimetic membrane system.

Bars show mean ± SEM (n = 3 coverslips analyzed per condition; differences between groups: **p < 0.01; two-way ANOVA). See also Figure S2.
Figure 3. CNP Associates with and Organizes the Actin Cytoskeleton to Reinforce its Antagonistic Function in Membrane Compaction by MBP

(A) F-actin in vitro co-sedimentation assay with full-length CNP (fl-CNP) and two constructs of the catalytic domain (CNPcat; CNPcat+C, an extended version of CNPcat by 22 residues). The position of actin is indicated by the black arrowhead in (A), (B), and (D). For comparison, equal fractions of the supernatant (S) and pellet (P) were loaded onto the gel in panels (A), (B), and (D). Please note that fl-CNP and actin are not separated in the gel system used here, good separation can be seen in Figure S2.

(B) Co-sedimentation of the CNP N-terminal domain (CNP-N) and the C-terminal catalytic domain (CNPcat). Both domains appear to co-sediment independently with F-actin.

(C) Electron micrograph showing negatively stained actin bundles that are decorated with full-length CNP stained with immunogold/monoclonal anti-His. Scale bar, 200 nm.

(legend continued on next page)
Bars show mean ± SD; difference between the groups: ***p < 0.001; two-way ANOVA.

(I) Quantification of GUV bursting over time. Bars show mean ± SD; difference between the groups: ***p < 0.001; two-way ANOVA.

(J and K) Electron micrographs of high-pressure-frozen optic nerves of ADF/cofilin1 double-knockout mice (P75) and as a control, wild-type C57BL/6J (K) and control animals (CNP-Cre/cofilin flox/flox) (J).

(L) Quantification of the number of axons with myelin sheaths containing cytoplasmic regions in cross sections. Bars show mean ± SD (n = 3; 350–470 axons per animal; **p < 0.01; t test). See also Figure S3.

To obtain further evidence for a role of F-actin in stabilizing of cytoplasmic-rich areas, we performed experiments in primary cultures of oligodendrocytes. We have previously shown that these cultures satisfy many of the essential requirements necessary to study the formation of compacted myelin, as they resemble in vivo compact myelin in composition (Aggarwal et al., 2013). Cultured oligodendrocytes develop membrane sheets that contain compacted membranes enriched in MBP and cytoplasm-rich regions with F-actin (Nawaz et al., 2015; Zuchero et al., 2015). To determine the role of F-actin in stabilizing the cytoplasmic regions within the sheets, we depolymerized actin with latrunculin A or cytochalasin B and quantified the area of the sheets covered by MBP after treatment (Figure S3). We found that both drugs led to an increased area covered and compacted by MBP in the sheets, indicating that F-actin had blocked membrane compaction as previously shown (Dyer and Benjamins, 1989).

To determine whether increasing F-actin levels reduce the area of the sheets covered by MBP, we analyzed primary cultures from mice that specifically lack cofilin1 and actin depolymerizing factor (ADF) in oligodendrocytes (Adf1−/−, Cnp1Cre/WT; Cfl1flox/flox, also termed ADF/cofilin1 double knockout) as a consequence have elevated levels of F-actin. Indeed, when primary cultures of mutant oligodendrocytes were prepared, we found that membrane sheets contained fewer MBP-rich regions (Figure S3). To obtain in vivo proof of this finding, we analyzed high-pressure frozen optic nerves of ADF/cofilin1 double-knockout mice. We found significantly more cytoplasmic regions in myelin of ADF/cofilin1 double-knockout mice (P15) than in controls (Figures 3J–3L), indicating that F-actin levels contribute to the formation of cytoplasmic regions within myelin sheaths.

**Rescue of Large-Caliber Axons in CNP-Deficient Mice by Reducing MBP Levels**

We hypothesized that intact cytoplasmic channels are necessary for maintaining functional axon-myelin units. Since CNP-deficient mice exhibit progressive axonal pathology with amyloid precursor protein (APP)-positive swelling and spheroid formation (Edgar et al., 2009; Lappe-Siefke et al., 2003), we asked whether the axonal degeneration phenotype is rescued by reducing MBP levels. Indeed, when cross sections of the fimbria in CNP null/shiverer heterozygotes (P75) were compared to CNP-deficient mice, we noticed the complete rescue from APP+ spheroids (Figures 4D–4G). The axonal degeneration in CNP-deficient mice is accompanied by activation of microglial cells, possibly to clear the damaged axons (Lappe-Siefke et al., 2003). When immunolabeled for the microglial marker Mac3, reduced MBP expression was associated with a lower number of microglia in CNP mutants (Figure S4). Hence, lowering MBP in CNP-deficient mice results in less axonal damage, which is also reflected by a reduced microgliosis.

Next, we performed electron microscopy and found that at age P60 and P180, when axonal degeneration could be observed in CNP-deficient mice (Figures 4H and 4I), the degree of axonal degeneration and pathological myelin outpacing...
in the spinal cord of CNP null/shiverer heterozygotes was significantly reduced (Figure 4). Importantly, when we analyzed the axonal pathology at the later time point, the axon-protective effect of reduced MBP expression on CNS axons was maintained at least until P180 (Figure 4). Finally, we asked whether the protective effect includes thin-caliber axons of the optic nerve that contain myelin with little cytoplasm. In contrast to the spinal cord, axonal degeneration in the optic nerve was not alleviated in Cnp null/shiverer heterozygous mice (Figure 4J). Taken together, our findings provide evidence for a role of CNP in maintaining cytoplasmic channels in myelin, a function important for maintaining axonal integrity of large-caliber CNS axons in mice.

**DISCUSSION**

In this work, we used high-pressure freezing to improve tissue preservation of CNS white matter tracts and to elucidate myelin structure close to its native state. Using this technique, we identified CNP as an essential protein in setting up and maintaining normal cytoplasmic regions within myelin sheaths. At the molecular level, we find that CNP acts together with F-actin to antagonize the membrane adhesive forces exerted by polymerizing MBP molecules. One model of how CNP could exert such a function is by forming pillars anchored to the membrane by the actin cytoskeleton in the cytoplasmic space of the myelin sheath. Such CNP struts could keep the cytoplasmic leaflets separated, thereby preventing membrane compaction by MBP. Keeping these spaces open is likely to allow a more efficient diffusion of metabolites and enable the motor-driven transport of vesicular cargo. Thus, two antagonistic molecular forces appear to operate in myelin: one depending on MBP and the other based on CNP and the actin cytoskeleton. It is possible that there is a “tug-of-war”-type regulation of a cytoplasmic compartment within myelin in which the actin cytoskeleton in association with CNP prevents MBP from compacting the membrane multilayer (Figure 4K). This model is in good agreement with the observation of more non-compacted myelin in transgenic mice overexpressing CNP (Gravel et al., 1996).

What is the function of cytoplasmic channels in adult myelin? Our analysis now shows that cytosolic channels remain a prominent compartment of (non-compact) myelin around large-caliber axons in the adult. These findings are consistent with previous studies, which have been able to visualize cytoplasmic channels in thick myelinated fibers of adult spinal cord using dye injections (Velumian et al., 2011). However, cytoplasmic channels were also identified after myelination (P30) in oligodendrocytes of rat optic nerve (Butt and Ransom, 1993) and in cortical myelin of adult mice (Murtie et al., 2007). In the peripheral nervous system, microtubules, actin, and mitochondria have been documented in the cytoplasmic pockets (or Schmidt-Lanterman incisures) within compacted myelin (Hall and Williams, 1970). It is therefore tempting to speculate that they are required for the transport of molecules across the myelin sheath to the axon and for providing plasticity to the myelin sheath. Consistent with this concept, CNP-deficient mice have not only a reduced number of cytoplasmic regions but also ongoing axonal pathology, with axonal swelling and spheroid formation (Edgar et al., 2009; Lappe-Siefke et al., 2003). Here, the frequently observed enlargement of inner axonal tongues, filled with granular material (Lappe-Siefke et al., 2003), can be explained by the traffic block within cytoplasmic channels and the backlog of cytosolic cargo that leads to secondary swelling of the inner tongue and paranodral abnormalities (Rasband et al., 2005).

Importantly, we observed that by reducing MBP levels in CNP-deficient mice, cytoplasmic channels became more prominent again and axonal pathology was rescued in large-caliber axons. The rescue of axonal integrity was not seen in thin-caliber axons, possibly because oligodendrocytes that generate myelin around thin-caliber axons have shorter internodes (Bechler et al., 2015).

In summary, we can propose a model for a molecular mechanism by which the cytoplasmic compartment is regulated in size and maintained in myelin sheaths. Our study provides a molecular and structural framework for understanding how myelin is kept “alive” and metabolically active and how oligodendrocytes remain functionally connected to the axonal compartment. We hypothesize that a system of cytoplasm-rich channels, bidirectionally connecting the oligodendrogial cell body with the inner adaxonal tongue of myelin, are necessary to provide metabolic support, maintain functional axon-glial units over a long period of time, and regulate myelin thickness within active neuronal circuits.

**EXPERIMENTAL PROCEDURES**

**Electron Microscopy**

Mice were killed by cervical dislocation, and freshly extracted optic nerves and spinal cords were cryo-fixed using a high-pressure freezer HPM100 (Leica) and further processed by freeze substitution and EPON-embedding following the “tannic acid-OsO4 protocol” described in (Möbius et al., 2010). Prior to the freezing of the cervical spinal cord samples were immersion fixed for 5 min in 4% PFA and 2.5% GA followed by vibratome sectioning (VT 1200, Leica) in 200-μm slices. These slices were then high-pressure frozen. Ultrathin cross-sections of myelin were prepared, and these sections were post-fixated and stained with uranyl acetate and lead citrate for electron microscopy.

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Cell Reports 18, 314–323, January 10, 2017 321
sections were obtained with a Ultract U ultramicrotome (Leica) and contrasted as described previously (Möbius et al., 2010). Sections were imaged using a LEO 912 Omega electron microscope (Zeiss) equipped with an on-axis 2k charge-coupled device (CCD) camera (TRS). Three to five animals were used for each analysis. On cross sections, 5–15 randomly selected areas of 150 μm² were imaged per animal in which 100–300 myelinated axon profiles with four or more myelin wraps were assessed.

**Immunohistochemistry**

For immunohistochemistry, antibodies specific for amyloid precursor protein (APP; 1:1,500, Chemicon), gial fibrillary acidic protein (GFAP; 1:200, Novocastra), and MAC3 (1:400, BD Pharmingen) were used. Four or five male mice per genotype (blinded to the genotype) were analyzed at P75. Per marker and mouse, one histological section comprising both fimbriae were analyzed, and the mean of both fimbriae was used for statistical assessment.

**Statistical Analyses**

Statistical analysis was performed using Excel (Microsoft) and GraphPad Statistical Analyses.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.12.053.

**AUTHOR CONTRIBUTIONS**

N.S. and C.V. designed and performed experiments, analyzed the data, and wrote the manuscript. W.M. assisted with the electron microscopy experiments and data analysis. H.B.W. analyzed data. M.S.E., M.M., A.R., and A.I. performed experiments and analyzed the data. K.-A.N., P.K., and M.S. designed experiments, supervised the research, and wrote the manuscript.

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