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A neuronal PI(3,4,5)P3-dependent program of oligodendrocyte precursor recruitment and myelination

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The molecular trigger of CNS myelination is unknown. By targeting *Pten* in cerebellar granule cells and activating the AKT1/mTOR pathway we increased the caliber of normally unmyelinated axons and the expression of numerous genes encoding regulatory proteins. Surprisingly, this led to the expansion of genetically wildtype OPCs, oligodendrocyte differentiation, and the *de novo* myelination of parallel fibers. Thus, a PI(3,4,5)P3-dependent neuronal program is sufficient to trigger all steps of myelination.

In the peripheral nervous system, the 'trigger' of myelination is axon size ($>1\mu\text{m}$), a threshold mediated by Nrg1 type III on the axon surface¹. Since Nrg1 is not required for CNS myelination², we asked whether axon size can be experimentally increased here by the absence of PTEN³, i.e. with PI(3,4,5)P3 stimulating the AKT1/mTOR pathway (Fig.1a). To specifically enlarge cerebellar granule cells (GC) and their naturally unmyelinated parallel fiber (Pf) axons, we generated *Tg(mα6)-Cre*Pten^{loxP/loxP}* mice^{4, 5}. By reporter gene expression⁴, recombination begins at P9 in predominantly postmitotic GC. The cerebellum of *Tg(mα6)-Cre*Pten^{loxP/loxP}* mice (hereafter termed *Pten* cKO or 'mutants') revealed a loss of *Pten* mRNA (-63%; Fig.1b) and protein (-80%; $n=3$ each genotype; $p=0.0025$, $t=6.788$, $d.f.=4$, two-tailed unpaired Student's t-test; Fig.1c) compared to *Pten^{loxP/loxP}* mice (hereafter termed 'controls') at age 3.5 months. Loss of PTEN resulted in enhanced phosphorylation of well-known downstream targets of the PI3K pathway (Fig.1d).

Mutant mice appeared healthy (Supplementary Video1), but cerebellar GC layer (GL) and molecular layer (ML) progressively enlarged (Fig.1e; Supplementary Fig.1a). Immunostaining of GABA_A receptor α6 revealed a gradual size increase of GC somata (Supplementary Fig.1b). Older mice developed ataxia and tremor (Supplementary Video2), possibly due to hamartomas at advanced age (Supplementary Fig.2; Supplementary Information).

The diameter of Pf also increased over time, as quantified by electron microscopy (EM), reaching $0.61\pm0.009\mu\text{m}$ in mutants over $0.16\pm0.002\mu\text{m}$ in controls at 1 year of age (Fig.1f,g; Supplementary Fig.1c). Importantly, radial Pf growth in mutant brains was associated with significant myelination beginning at age P40 ($2.3\pm0.6\%$ myelinated Pf) and progressing over time (3 months: $9.5\pm1\%$ myelinated Pf), up to

40±3% of Pf being myelinated at 1 year with an average g-ratio of 0.84. Myelination was visualized by Gallyas silver impregnation (Supplementary Fig.3a), anti-CNP immunohistochemistry (Fig.1h, Supplementary Fig.3b), including whole-mount immunolabelling combined with light sheet and 2-photon microscopy (Supplementary Video3), as well as EM (Fig.1i-k). We determined a 'size threshold' of approximately 0.25 µm for myelination at all ages tested (Fig.1k,l). Myelinated Pf segments were very rarely seen in controls.

De novo myelination included the formation of nodes and paranodes with septate-like junctions (Fig.1j) and the clustering of Caspr and Nav1.6 on axons (Fig.1m,n). GC synaptic contacts to Purkinje cell dendrites were restricted to Pf 'nodal' regions (Fig.1o; Supplementary Fig.4).

The ML harbors normally very few oligodendrocytes and only scattered OPCs, as demonstrated in *Plp1-DsRed*Ng2-EYFP* double-transgenic mice (Fig.2a). In *Pten* mutants at P45, mature oligodendrocytes were increased in the GL (+33%) and more prominent in the ML (+750%), yet unaltered in cerebellar white matter (WM) (Fig.2b). To determine whether OPCs proliferate in the ML or are recruited from the GL, we combined immunostaining with BrdU labeling (Fig.2c). After 20 days of daily BrdU injections (P25-45), OPC proliferation (BrdU+,Olig2+) was the same in GL or WM of mutants and controls (Fig.2d). However, OPC proliferation in the mutant ML was 4.4-fold increased. Even at age 6-7 months, we found a 4.7-fold increase of BrdU+,Olig2+ cells. This suggests that local proliferation is stimulated (directly or indirectly) by GC axon-derived signals, which must still be present at older ages. At that time, oligodendrocyte lineage cells were 10-fold increased over controls (Fig.2d). By following different BrdU protocols we identified a time window (P20-30) at which proliferation of OPCs was already increased, but the number of (CNP+) oligodendrocytes was not yet altered (Fig.2e,f). This strongly suggests that OPC proliferation is not merely a homeostatic mechanism to replace differentiated OPCs, but is an early response to the neuronal trigger. Accordingly, (BrdU+,Olig2+) OPCs were uniformly distributed in the ML and not preferentially detected in proximity to newly generated (MBP+) oligodendrocytes (Fig.2g).

Double-labeling for BrdU and carbonic anhydrase II (CAII), a marker of mature oligodendrocytes, confirmed that newly generated OPCs had responded to elevated

differentiation signals (Fig.2h). At 7 months, the ML in *Pten*-mutant brains still contained 'maturing' oligodendrocytes, as revealed by *in situ* hybridization of *Myrf* mRNA (Fig.2i), an early marker almost undetectable in fully mature cells (B. Emery, personal communication). Taken together our data suggest that *Pten*-mutant GC and thus a PI(3,4,5)P3-dependent neuronal program is sufficient to drive oligodendrocyte lineage cells through all developmental stages. However, we cannot formally exclude the theoretical possibility that normally myelinated axons recruit OPCs and induce myelination by completely different mechanisms. Formally, activation of neuronal Akt1/mTOR would not be the "initiating" event itself, if other more rate-limiting steps are regulated upstream.

OPCs receive synaptic input that could modulate proliferation and myelination⁶. Using acute slices from mutant and control mice (additionally expressing a *Ng2-EYFP* transgene⁷), we whole-cell patch clamped both Purkinje cells, having input predominantly from Pf, and fluorescent OPCs in the ML. This allowed us to determine GC spiking activity as well as the synaptic inputs from Pf to OPCs. Importantly, we found no difference between mutant and control OPCs for the frequency of spontaneous synaptic Pf inputs, nor the inputs to Purkinje cells (Fig.2j). Also the fraction of OPCs that received detectable input was not different (controls: 39%; mutants: 47%; $\chi^2=0.87$, $p=0.4$, Chi-square test). Thus, *de novo* myelination is unlikely owed to an underlying alteration of Pf spiking activity or changes of synaptic input to OPCs (Supplementary Fig.5; Supplementary Information).

Theoretically, Pf myelination could be caused by down-regulating inhibitory neuronal signals. We addressed this by targeting only a small fraction of sparsely distributed GC, using *Nex-CreERT2* mice⁸. After 5 days of tamoxifen (P10-15), reporter gene expression marked <4% of all GC (Supplementary Fig.6a). However, when analyzed 28 weeks later (Supplementary Fig.6b), we still found a significant increase of mature OL and myelinated area in the ML of *Nex-CreERT2* **Pten*^{loxP/loxP} mice (Supplementary Fig.6c-f), making it highly unlikely that soluble inhibitors, still expressed by 96% of non-recombined GC, must be down-regulated to initiate myelination. In these mice *de novo* myelination was also observed when tamoxifen was given at age 7 months (not shown), arguing strongly against a critical period in development at which the identified PI(3,4,5)P3-dependent neuronal program can trigger myelination.

Searching for promyelinating factors, we laser captured stripes of GL from cryosections of 3 months old mutants and controls for transcriptome analyses (Fig.3a). A resulting list of candidate genes, upregulated in *Pten* cKO, was further selected by annotation (Supplementary table1) and validated with qPCR (not shown).

To confirm the significance of these findings *in vivo*, we tested Bdnf⁹, reasoning that its inactivation might affect *de novo* myelination. Surprisingly, *Tg(mα6)-Cre*Pten^{flox/flox}*Bdnf^{flox/flox}* mice (hereafter termed *Pten*Bdnf* double mutants) exhibited a higher (not lower) number of oligodendrocytes in the ML compared to *Pten* single mutants (Fig.3b,c). Also by immunostaining, the myelinated area of the ML was not smaller but larger (Fig.3d,e). By EM, we confirmed that Pf calibers were as enlarged as in *Pten* single mutants (Fig.3f). However, by g-ratio analysis myelin was thinner in *Pten*Bdnf* double mutants (Fig.3g,h). Thus, in the absence of Bdnf, mutant oligodendrocytes are more numerous in the ML, but each fiber is only thinly ensheathed. Most likely, OPCs respond to either proliferation or differentiation signals, but not to both. In the absence of Bdnf (a differentiation signal) the weight of proliferation signals might increase, driving the number of OPCs up. However, differentiation signals will compensate later and turn all OPCs into oligodendrocytes, explaining a seemingly paradoxical result.

To test this hypothesis directly, we compared OPC proliferation in *Pten*Bdnf* double mutants and single mutants at only 7 weeks of age, following BrdU labeling (P25-45). Indeed, double mutants showed 34% more BrdU-labeled (Olig2+) OPCs in the ML than *Pten* mutants (Fig.3i). Surprisingly, the lack of Bdnf even promoted proliferation of OPCs in heterozygous *Pten* mutants compared to controls (Fig.3i). This suggests that Bdnf helps controlling the number of OPCs, which are likely to turnover in the absence of other myelination promoting factors. For comparison, Nrg1 (essential for myelination by Schwann cells¹) revealed no such effects on *de novo* myelination in *Tg(mα6)-Cre*Pten^{flox/flox}*Nrg1^{flox/flox}* mice (Supplementary Fig.7).

Because of the complexity of Bdnf effects *in vivo*, we studied other candidate factors (emerging from our screen) in primary oligodendrocyte cultures and myelinating neuron/oligodendrocyte co-cultures, using recombinant proteins to test their role in OPC proliferation, differentiation, and/or myelination. Indeed, Fgf1 stimulated

proliferation of OPCs (Supplementary Figs.8a-d and 9; Supplementary Information). Activin-A, the biologically active dimer of two inhibin betaA (Inhba) subunits, emerged as a differentiation factor¹⁰ that required MAPK (ERK1/2) to be effective, but not PI3K or SMAD3 (Supplementary Fig.8e-i; Supplementary Information). Also Pleiotrophin, Tmsb4x and Timp3 stimulated *in vitro* myelination (Supplementary Fig.8g,h; Supplementary Information).

Increased axon size was further associated with upregulated expression of several other genes. Some mRNAs encoded secreted proteins already associated with oligodendrocyte development *in vitro*, such as Bdnf, Vegfc, and Tmsb4x^{9,11,12}. Other factors, including Fstl1, IGFBP-4, Dkk-3, Apcdd1, and Sfrp4, are novel, but known antagonists of Wnt and Bmp signaling^{13,14}, which inhibits oligodendrocyte differentiation¹⁵. Also modulators of IGF signaling and metallopeptidases (MMP17, Adamts1) were upregulated, the latter degrading inhibitors of remyelination. We note that also membrane proteins not transcriptionally upregulated may increase in abundance when axons grow radially.

Taken together, we have identified an upstream neuronal "switch", the PI3K/AKT1/mTOR pathway, which is sufficient to trigger the entire program of radial axonal growth¹⁶, recruitment of OPCs and progressive myelination in the CNS. Axonal specializations, such as the clustering of Caspr at paranodal junctions, are part of this neuronal myelination program. Clearly, myelination is triggered by a combination of both, the increase of axon caliber (>0.25µm) and the induced expression of numerous promyelinating factors. Axon caliber poses a critical threshold for myelination in PNS and CNS, as recently shown for cultured oligodendrocytes myelinating carbon nanofibers¹⁷. However, CNS myelination begins with OPC recruitment and expansion, followed by oligodendrocyte differentiation, all steps without axonal contact. Only once differentiated oligodendrocytes physically engulf axons, the minimum caliber is required to allow membrane wrapping. Schwann cells, in contrast, are always physically associated with axons and stimulated by a key membrane-bound growth factor, Nrg1 type III¹.

Down-stream of the neuronal myelination switch, not all promyelinating factors are of neuronal origin, in line with previous findings¹⁰. We also identified immune

modulatory factors upregulated, such as Nts (neurotensin) and Ninj1, previously implicated in immune cell migration^{18,19}. Since moderate changes of Iba1, Mac-3, GFAP were detected in the mutant ML (Supplementary Fig.10a-c), we asked whether non-neuronal cells respond to *Pten* deletion in *Nex-CreERT2*Pten^{flax/flax}* mutants (only 4% GC recombination but significant myelination). Here, no 'gliosis' markers were detected in ML and GL, even 28 weeks after recombination (Supplementary Fig.11). Thus, *de novo* myelination is not dependent on any form of 'gliosis'.

Moreover, several factors are expressed in endothelial cells (Supplementary table1) and by CD31 immunostaining we noticed increased angiogenesis in the mutant GL (Supplementary Fig. 10d). This is in line with the recently identified coupling of myelination to angiogenesis²⁰.

Importantly, not all *Pten* mutant neurons become myelinated as evidenced by hippocampal neurons in *Nex-CreERT2*Pten^{loxP/loxP}* mutants. Despite efficient recombination of CA3 neurons⁸ and significant increase in the size of neuronal somata, Schaffer collateral axons remained unmyelinated (Supplementary Fig.12). This suggests a role of inhibitory signals that may 'protect' axons from myelination, which would interfere with sprouting and neuronal plasticity in the adult. However, we have no formal proof that all axons were sufficiently enlarged, as EM sections did not allow us to positively identify the Schaffer collaterals.

In multiple sclerosis, lesioned areas fail to repair, presumably because of a poor balance of myelination promoting and inhibiting cues. Significant progress has been made in identifying small molecules targeting OPCs and oligodendrocytes in remyelination. Our data suggest that also neurons should be considered as therapeutic targets when searching for myelin repair strategies.

Accession Codes

The following ImageJ plugins were used and can be freely accessed under:

http://www1.em.mpg.de/Goebbels2016_MBP

http://www1.em.mpg.de/Goebbels2016_GFAP

http://www1.em.mpg.de/Goebbels2016_CoCultureMBP

http://www1.em.mpg.de/Goebbels2016_CoCultureSmi31

http://www1.em.mpg.de/Goebbels2016_CoCultureOlig2

http://www1.em.mpg.de/Goebbels2016_CoCultureDapi

Data Availability

GEO: microarray data, accession code: GSE80966.

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Author contributions:

S.G. and K.A.N. developed study concept and design. A.P., B.W., O.Y., J.M.E., K.K., and G.L.W. performed immunoblotting, immunohistochemistry, cell culture analyses and electron microscopy under supervision of S.G.. K.Y. performed in situ hybridization. N.R. performed volume imaging under the supervision of M.T.L.. G.L.W. and S.P.W. performed laser capture microdissection and subsequent microarray hybridization under supervision of M.J.R.. S.S. performed electrophysiological recordings under supervision of R.T.K.. A.A. generated *Nex-CreERT2* mutant mice. S.G., G.L.W., R.T.K., and K.A.N. wrote the manuscript. All authors have contributed to and approved the manuscript.

Competing Financial Interests Statement:

The authors declare no competing financial interests.

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Figure Legends

Figure 1:

***Pten* mutant GC trigger *de novo* myelination of Pf axons**

(a) Schematic representation of selected candidates of PI3K/AKT1/mTORC1 signaling.

(b) By qRT-PCR *Pten* transcripts containing the floxed exon 5 are decreased in the cerebellum of conditional *Pten* mutants (age 3.5 months). Means $\pm$ s.e.m. n=5 mice each genotype (p=0.0231, t=2.803, d.f.=8, two-tailed unpaired Student's t-test).

(c) On Western blots PTEN levels are decreased in the cerebellum of mutants (age 3.5 months, **cropped blot images**).

(d) Phosphorylation (p-) of the PI3K downstream effectors AKT1, GSK3 β , mTOR and S6 is enhanced in *Pten* mutant mice (age 3.5 months, **cropped blot images**).

(e) Conditional ablation of *Pten* in GC causes cerebellar enlargement.

(f) Pf have increased in diameter when analyzed by EM.

(g) Pf caliber size distribution (n=3 mice each genotype; 140 Pf quantified per animal).

(h) Myelinated Pf in the ML of mutant brains (right) immunostained for CNP (green). Purkinje cells (PC) and interneurons (IN) are Parvalbumin positive (red). Arrowhead points to an oligodendrocyte (OL) cell body. PL, Purkinje cell layer.

(i) By EM normally unmyelinated Pf become *de novo* myelinated in mutant mice (asterisks).

(j) Formation of a node of Ranvier (N), flanked by paranodal myelin loops (Pn; left panel) and septate-like junctions (right panel) with the Pf membrane at the EM level.

(k) Progressive myelination quantified by g-ratio analysis at the indicated ages (g=1.0 denotes unmyelinated axons, n=3 mice each genotype and age, 140 Pf quantified per animal).

(l) *De novo* myelination of Pf requires a minimum axonal caliber of 0.25 μ m.

(m,n) Myelination of Pf includes the clustering of axonal Caspr (**m**, green; **n**, red) and Nav1.6 (**n**, green) at newly formed nodes.

(o) Myelinated Pf synapsing on a Purkinje cell dendritic spine. "En passant" synapses with presynaptic vesicles (arrowhead) are restricted to newly established nodes.

M, myelin; Pf, parallel fiber; Pn, paranodal loop; s, dendritic spine. **e-j** and **l-o**, age 1 year. Images in **e,f**, **h-j** and **l-o** are representative of ≥ 3 similar experiments.

Figure 2:

***Pten* mutant GC drive OPC proliferation in the ML**

(a) Oligodendrocytes (in red) are found in cerebellar GL but not in the ML, which contains only scattered NG2⁺ OPCs (green). Cells are labeled by DsRed and EYFP fluorescence in double-transgenic *Plp1-DsRed*Ng2-EYFP* mice.

(b) Mature oligodendrocytes (CAII⁺) are more numerous in the mutant GL ($p=0.0498$, $t=2.780$, d.f.=4) and ML ($p=0.0103$, $t=4.562$, d.f.=4, inset), but not in WM ($p=0.858$, $t=0.1907$, d.f.=4). Age P45; $n=3$ each genotype.

(c) OPCs in the mutant ML labeled for Olig2⁺ (red) and BrdU⁺ (green, arrowheads), following daily BrdU administration between P25-P45 (quantified below).

(d) At age P45, proliferation of OPCs (BrdU⁺, Olig2⁺) is increased in the mutant ML ($p=0.0004$, $t=25.49$, d.f.=4), and remains elevated at P201 (following daily BrdU administration between P181-P201; $p=0.0016$, $t=10.98$, d.f.=4). Note that the number of Olig2⁺ cells at P201 is 10-fold higher than in controls ($p=0.0031$, $t=11.37$, d.f.=4). WM, $p=0.8581$, $t=0.1907$, d.f.=4; GL, $p=0.4133$, $t=0.9122$, d.f.=4; $n=3$ each genotype and age.

(e,f) Following daily BrdU administration (P15-P20) the number of proliferating OPCs (BrdU⁺, Olig2⁺) in the ML is similar in mutants and controls (in **e**; $p=0.3161$, $t=1.145$, d.f.=4). While at age P30 (and after daily BrdU administration between P20 and P30) the number of proliferating OPCs (BrdU⁺, Olig2⁺) in the ML is increased in the mutants (left panel in **f**; $p=0.0120$, $t=4.364$, d.f.=4), the number of CNP⁺ oligodendrocytes is not (right panel in **f**, $p=0.8025$, $t=0.2673$, d.f.=4). $n=3$ each genotype and age.

(g) Following daily BrdU administration (P25-P45) labeled OPCs (asterisks) distribute evenly and are not preferentially close to newly generated oligodendrocytes and their myelin sheaths (arrowheads).

(h) After daily BrdU injections (P25-P45) mature oligodendrocytes can be co-labeled for BrdU (green) and CAII (red). $n=3$ each genotype ($p=0.0006$, $t=9.839$, d.f.=4).

(i) By *in situ* hybridization, *Myrf* mRNA can still be detected in the adult *Pten* mutant ML (positive control, wildtype cerebellum P20).

(j) Representative traces of spontaneous postsynaptic inward currents recorded from NG2-EYFP expressing OPCs clamped at -74mV in the ML of (a) control mice and (b) *Pten* mutant mice (a', b', inward currents at an expanded time scale); (c) frequency of detectable spontaneous events in OPCs plotted against the age of the animal. The slopes are not significantly different between control (blue, $n=42$ cells from 25 animals of 12 different litters) and mutant mice (red, 28 cells from 17 animals of 11 litters) ($p=0.2$, $F=1.625$, d.f.=66, linear regression). (d) Similarly, there is no significant difference in the frequency of spontaneous inputs in Purkinje cells (PC) which are predominantly from Pf (control, 0.9 ± 0.1 Hz, $n=5$ cells from 2 animals of 1 litter; mutant, 0.7 ± 0.2 Hz, $n=4$ cells from 1 animal of 1 litter; $p=0.36$, $t=2.13$, d.f.=10).

Data are means $\pm$ s.e.m. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed unpaired Student's t-test (in **b,d,e,f**, and **h**), chi-square test (in **jd**), Analysis of covariance (ANCOVA) (in **jc**). GL, GC layer; ML, molecular layer; WM, white matter. Images in **a,c** and **g-i** are representative of 3 similar experiments.

Figure 3:

Screen for candidate promyelinating factors and validation of Bdnf

(a) Experimental design to identify and test candidate factors (see Supplementary Information) that may regulate oligodendrocyte development in conditional GC-specific *Pten* mutant mice. Laser capture microdissection (LCM) was used to obtain the GL from serial cerebellar sections at age 3 months.

(b-e) Double mutants lacking both PTEN and Bdnf in GC harbor more CNP+ oligodendrocytes in the ML (arrowheads in **b**, **c**: 7 weeks, control vs. *Pten* het**Bdnf* cKO, $p=0.0495$, $t=2.786$; control vs. *Pten* cKO, $p<0.0001$, $t=28.25$; *Pten* cKO vs. *Pten* cKO**Bdnf* cKO, $p=0.0132$, $t=3.757$, d.f.=13, $F=56.01$; 10 weeks, control vs. *Pten* het**Bdnf* cKO, $p=0.1530$, $t=1.684$; control vs. *Pten* cKO, $p<0.0001$, $t=55.87$; *Pten* cKO vs. *Pten* cKO**Bdnf* cKO, $p=0.0012$, $t=6.661$, d.f.=13, $F=1895$) and exhibit a larger MBP+ myelinated area (**d**, **e**: 7 weeks, control vs. *Pten* het**Bdnf* cKO, $p=0.4886$, $t=0.7619$; control vs. *Pten* cKO, $p=0.0085$, $t=4.196$; *Pten* cKO vs. *Pten* cKO**Bdnf* cKO, $p=0.0024$, $t=5.021$, $F=40.64$, d.f.=13; 10 weeks, control vs. *Pten* het**Bdnf* cKO, $p=0.3938$, $t=0.9547$; control vs. *Pten* cKO, $p=0.0039$, $t=5.979$; *Pten* cKO vs. *Pten* cKO**Bdnf* cKO, $p=0.0476$, $t=2.611$, $F=23.02$, d.f.=12) compared to single *Pten* mutants (quantitated on parasagittal sections of the cerebellar vermis; $n=3-4$ each genotype at indicated ages).

(f) By EM of *Pten***Bdnf* double mutants and single *Pten* mutants, axon sizes are unchanged in both myelinated ($p=0.4339$, $t=0.869$, d.f.=4) and unmyelinated Pf ($p=0.5558$, $t=0.642$, d.f.=4; age 10 weeks; $n=3$ each genotype).

(g-h) G-ratio analysis of ML myelin in *Pten* single mutants and *Pten***Bdnf* double mutants ($p=0.0259$, $t=3.454$, d.f.=4; 250-490 myelinated Pf per animal; $n=3$ each genotype; age, 10 weeks).

(i) BrdU administered from P25-P45 and histology performed 4 days later. The number of BrdU+, Olig2+ cells is higher in *Pten***Bdnf* double mutants compared to *Pten* single mutants (control vs. *Pten* het**Bdnf* cKO, $p=0.0419$, $t=2.74$; control vs. *Pten* cKO, $p<0.0001$, $t=16.53$; *Pten* cKO vs. *Pten* cKO**Bdnf* cKO, $p=0.0426$, $t=2.565$, $F=56.01$, d.f.=13; quantified in the ML of parasagittal sections of the cerebellar vermis; $n=3-4$ each genotype).

Data are means $\pm$ s.e.m. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-tailed unpaired Student's t-test (in **f,h**) or one-way analysis of variance (ANOVA) followed by Bonferroni test (in **c,e**, and **i**). GL, granule cell layer; ML, molecular layer. Images in **a,b** and **d** are representative of ≥ 3 similar experiments.

Online Methods

Mouse mutants

All experiments were conducted according to the Lower Saxony State regulations for the use of experimental animals in Germany as approved by the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES) and according to UK government animal use regulations. Mice were housed in groups in individually ventilated cages under a 12:12h light/dark cycle with access to food and water *ad libitum*. If not stated otherwise, for molecular, histological, electrophysiological, electron microscopical and biochemical experiments, female and male mice were included in the experiments and were randomly allocated to experimental groups according to age and genotype. All animal experiments were conducted in a single blinded fashion towards the investigator. Inclusion/exclusion criteria were pre-established. Animals were excluded from the experiment when showing impaired health conditions not attributable to genotype or experiment or when the weight difference to the average group weight was larger than 10%. With respect to the outcome assessment, exclusion criteria were determined with Grubbs' test (ESD method), using the statistical software GraphPad (Prism). No animals or samples had to be excluded with these criteria in any of the experiments.

Mice mutant for *Pten*^{flox/flox}, *Tg(mα6-Cre)B1LFR*, *Nex-CreERT2*, *Nrg1*^{flox/flox}, *Bdnf*^{flox/flox}, *Plp1-DsRed*, *Rosa26-lacZ* and *Ng2-EYFP* were genotyped as described^{4,5,7,8,21-24}. *Tg(mα6-Cre)B1LFR*, *Nex-CreERT2*, *Nrg1*^{flox/flox}, *Bdnf*^{flox/flox}, *Plp1-DsRed*, *Rosa26-lacZ* and *Ng2-EYFP* mutants were on C57BL/6N background, whereas *Pten*^{flox/flox} mutants and thus all compound mutants harboring *Pten*^{flox} alleles were on mixed C57BL/6N-SV129 background. For genotyping, genomic DNA was isolated from tail biopsies (InvisorbSpin Tissue Mini kit, Invitex) according to the manufacturer's directions and subjected to routine PCR methods using the following primers:

Pten sense 5'-ACTCAAGGCAGGGATGAGC-3'
antisense 5'-CAGAGTTAAGTTTTGAAGGCAAG-3'
Tg(mα6-Cre)B1LFR sense 5'-TAGAGCATTAGGGTGGGAG-3'
antisense 5'-TGCCGCCTTTGCAGGTGTGCTTAC-3'
Nrg1 sense 5'-GCACCAAGTGGTTGCGATTGTTGCT-3'
antisense 5'-TCCTTTTGTGTGTGTTTCAGCACCGG-3'
Bdnf sense 5'-GTTGCGTAAGCTGTCTGTGCACTGTGC-3'
antisense 5'-CAGACTCAGAGGGCACTTTGATGGCTTG-3'
Plp1-DsRed sense 5'-CGCCGACATCCCCGACTACAA-3'
antisense 5'-GCGGCCGCTACAGGAACAGGT-3'
Rosa26-lacZ sense 5'-AAAGTCGCTCTGAGTTGTTAT-3'
antisense 5'-GCGAAGAGTTTGTCTCAACC-3'
Ng2-EYFP sense 5'-TGACCTTGGATTCTGAGC-3'
antisense 5'-CGCTGAACTTGTGGCCGTTTA-3'

BrdU labeling

Mice received 5'-bromo-deoxyuridine (BrdU, Sigma Aldrich) by daily intraperitoneal injections (50 µg/g of body weight) for 5, 10, or 20 consecutive days. Mice were sacrificed 4 hours or 4 days post final injection.

Histology and immunohistochemistry

Mice were anaesthetized with avertin (Sigma–Aldrich) and perfused with 15 ml of

Hanks balanced salt solution (HBSS, PAA laboratories, Pasching, Austria) followed by fixative (4% PFA, 0.1M Phosphate buffer, 0.5% NaCl). Paraffin sections of the brain (5 μ m) were deparaffinized using xylene and isopropanol and rehydrated in a descending ethanol series. Antigen unmasking was performed by boiling for 10–40 min in citric buffer, pH 6, or Tris EDTA, pH 9. Sections were blocked in 20% goat serum in PBS/BSA for 1 h and incubated with primary antibodies in 5% goat serum in PBS/BSA for 2 h at 37°C or overnight at 4°C. Detection was performed by using Alexa Fluor 488-, 555- and 633-conjugated secondary antibodies (1:1000, Thermo Fisher Scientific, #A-28175, #A-11034, #A-21212, #A-21422, #A-27039 and #A-21094) and by using biotinylated secondary antibodies, followed by diaminobenzidine (DAB) (LSAB2 Kit, Dako, #K0675; Vectastain Kit, Vector Laboratories, #BA-9400, #PK-6100). Primary antibodies were directed against BrdU (1:200, Chemicon, #MAB3424), CAIL (1:200, kindly provided by S. Ghandour, Strasbourg, PMID: 118210), Calbindin (1:600, Sigma, #C9848), Caspr (1:100, NeuroMab, #75-001), CD31 (1:100, Dianova, #DIA-310), CNP (1:150, Sigma, #C5922), GABA_A receptor α 6 subunit (1:500, Chemicon, #AB5610), GFAP (1:200, Novocastra, #NCL-GFAP-GA5), Iba1 (1:1000, Wako, #019-19741), MBP (1:200, Covance, #SMI-94R); Nav1.6 (1:500, Alomone Labs, #ASC-009); NeuN (1:200, Chemicon, #MAB377); Olig2 (1:200, kindly provided by J. Alberta, PMID: 15198128), and Mac-3 (1:500, BD Pharmingen, #553322). 5 μ m microtome sections (Microm HM400) were also stained by hematoxylin–eosin (HE) to study cytoarchitecture. Myelinated fibers were visualized by Gallyas silver impregnation as described²⁵. All images showing immunohistochemical analyses and stainings were successfully repeated at least three times. iDISCO whole-mount anti-CNP immunohistochemistry was performed according to the published protocol²⁶ on dissected cerebella from control and mutant animals. Monoclonal anti-CNP primary antibody (1:200, Sigma, #C5922) was used with a 4 days incubation at 37°C. Donkey anti-mouse Alexa Fluor 647-conjugated secondary antibody (1:1000, Life Technologies, #A-31571) was used for 4 days at 37°C. After clearing, samples were imaged on a LaVision Ultramicroscope II with a 2X 0.5NA objective and on an Olympus FV-1000 2 photon microscope with a 25X 1.05NA water immersion objective, and a laser tuned at 820nm. Image stacks were analyzed with Imaris 8.01 (Bitplane).

In situ hybridization

In situ hybridization was performed as described²⁷. A *Myrf* probe corresponding to 941bp from *Myrf* 3' UTR was kindly provided by B. Emery.

Electron microscopy

Mice were anaesthetized with avertin (Sigma–Aldrich) and perfused with 15 ml of Hanks balanced salt solution (HBSS, PAA laboratories, Pasching, Austria) followed by fixative (4% PFA, 0.1M Phosphate buffer, 0.5% NaCl, 2.5% glutaraldehyde). At parasagittal plane, cerebellar lobulus 5 was dissected, contrasted with 1% osmium tetroxide and embedded in epoxy resin. Semi-thin (0.5mm) and ultra-thin (50–60 nm) sections were cut, using a microtome (RM 2155, Leica Microsystems, Wetzlar, Germany) with a diamond knife (Histo HI 4317, Diatome, Biel, Switzerland). Semi-thin sections were stained with azur-II-methylenblue for 1 min at 60°C. Ultra-thin sections were stained with 2% uranylacetate (30 min) and 1% lead citrate solution (12 min) and analyzed using a LEO EM912AB electron microscope (Carl Zeiss NTS, Oberkochen, Germany). Images were taken with an on-axis 2048x2048 CCD camera (Proscan, Scheuring, Germany).

Electrophysiology

Parasagittal cerebellar slices (225 μm) were cut using a vibrating blade microtome (Leica VT1200S) from P7-P100 *Ng2-EYFP^{+/+}*Tg(ma6)-Cre*Pten^{loxP/loxP}* mice (mutants) and *Ng2-EYFP^{+/+}*Pten^{loxP/loxP}* mice (controls), culled by cervical dislocation in accordance with UK government animal use regulations. After dissection, the brain was placed in a cooled ($\sim 1^\circ\text{C}$) oxygenated (95% O_2 –5% CO_2) Krebs solution containing (mM): 126 NaCl, 24 NaHCO_3 , 1 NaH_2PO_4 , 2.5 KCl, 2.5 CaCl_2 , 2 MgCl_2 , 10 D-glucose (pH 7.4). In addition, kynurenic acid was included in order to block glutamate receptors, which might be activated during the dissection procedure and cause cell damage. During experiments slices were superfused at $22 \pm 1^\circ\text{C}$ with HEPES- buffered external solution containing (mM): 144 NaCl, 2.5 KCl, 10 HEPES, 1 NaH_2PO_4 , 2.5 CaCl_2 , 10 glucose, 0.1 glycine (to co-activate NMDA receptors), 0.005 strychnine (to block glycine receptors), pH set to 7.4 with NaOH, bubbled with 100% O_2 . OPCs were identified by EYFP expression and whole-cell voltage clamped. Recording electrodes had a resistance between 5–9 $\text{M}\Omega$ when filled with an internal solution comprising (mM): 130 K-gluconate, 4 NaCl, 0.5 CaCl_2 , 10 HEPES, 10 BAPTA, 4 MgATP , 0.5 Na_2GTP , 2 K-Lucifer yellow, pH set to 7.3 with KOH and the uncompensated series resistance was $40 \pm 1 \text{M}\Omega$ and electrode junction potentials ($\sim 14 \text{mV}$) were compensated for. A Multiclamp 700B (Molecular Devices) was used for voltage clamp data acquisition. Data were sampled at 50kHz and filtered at 10kHz using pClamp10.3 (Molecular Devices).

Synaptic current analysis

A synaptic current was defined to occur if its amplitude was bigger than twice the standard deviation of the baseline current noise and its 10–90% decay time was longer than its rise time. Events were detected and analyzed with pClamp 10.3 (Molecular Devices) and the Strathclyde Electrophysiology Software package WinEDR V3.3.7 WinWCP V4.6.2.

Laser-capture microdissection, RNA isolation and linear amplification, and microarray hybridization

Mice were killed at 3 months of age by cervical dislocation, total brains were dissected and frozen on dry ice, protected with parafilm and stored at -80°C . Serial coronal cryosections (20 μm) were prepared using a Leica CM3000 Cryostat, mounted on polyethylene naphthalate membrane frame slides (Arcturus), stained with thionin, and dried in an ascending ethanol/xylene series. For laser-capture microdissection (LCM), a Veritas Microdissection System (Arcturus) was used with slides mounted and stained the same day. Cerebellar GL were dissected by ultraviolet laser (laser power set to 3.2–4.2) and attached to CapSure LCM caps using an infrared laser (power set to 80%). Caps were collected in 0.5 ml Eppendorf tubes containing 100 μl of RNeasy Lysis Buffer (Qiagen) and stored at -80°C . Total RNA was isolated from LCM tissue using the Micro RNeasy Kit (Qiagen) according to the instructions of the manufacturer except that RNA was eluted from the column with 10 μl of water followed by precipitation with NaAcetate (pH 5.2, f.c. 0.3M) and PelletPaint (Novagen) as carrier. Two-round amplification and biotin-labeling, hybridization to mouse MOE230A 2.0 genechips (Affymetrix), washing and scanning was essentially as described²⁸ with 3 biological replicates for each genotype. Data analysis was performed using Genomics Suite (Partek Inc.). Raw data were normalized using the RMA algorithm and differentially expressed genes were identified with ANOVA according to the workflow suggested by the manufacturer (Partek Inc.). Differentially expressed genes with signal intensities in mutants >

controls were selected with >1.4-fold change and adjusted P-values <0.05.

RNA isolation, cDNA synthesis, and qRT-PCR.

Total RNA was isolated from LCM tissue using the Transcriptor High Fidelity Kit (Roche) according to the instructions of the manufacturer. cDNA was synthesized using a mixture of random nonamer primers and anchored poly-dT primers and SuperScript III RNase H reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. qRT-PCR was performed using SYBR green master mix (Applied Biosystems) and a 7500 Fast Real-Time PCR System (Applied Biosystems). Primers were designed using the Roche Universal ProbeLibrary website (<http://qpcr.probefinder.com>). Primer sequences are available upon request. Relative mRNA concentrations were normalized to Actin and Atp5b. Data were analyzed using qBase software version 1.3.5 (Center for Medical Genetics, Ghent University, Ghent, Belgium).

Mixed myelinating cocultures from mouse spinal cord

Wild-type C57BL/6N mice were time-mated, with the day of plugging denoted as embryonic day 0.5 (E0.5), and embryos were collected on embryonic day 13.5 (E13.5). The spinal cord was dissected, and dissociated mechanically and enzymatically (0.25% trypsin, Invitrogen). Enzymatic activity was stopped by the addition of plating medium (50% DMEM, 25% horse serum, 25% Hanks balanced salt solution without Ca²⁺ and Mg²⁺) containing 2.5 µg/ml DNase I. Cells were dissociated into a single cell suspension by triturating through 20- and 23-gauge needles (4 and 2 times, respectively) and spun at 800 rpm for 5 min. The pellet was resuspended in plating medium. Dissociated spinal cord cells were plated initially onto poly-L-lysine (PLL, in boric acid buffer, pH8.4) coated coverslips at a density of 150,000 cells/100 µl, which were then placed into a 35-mm Petri dish. The cells were left to attach for 2–3 hours, when 150 µl of plating medium and 500 µl of differentiation medium was added. This medium contained DMEM (4,500 mg/L glucose), 10 mg/ml biotin, 0.5% hormone mixture (1 mg/mL apotransferrin, 20 mM putrescine, 4 µM progesterone, and 6 µM selenium), 50 nM hydrocortisone, and 10 µg/ml insulin (all reagents were from Sigma). Cultures were maintained by replacing half of the medium with fresh medium three times a week. After 12 days in culture, cells were fed with differentiation medium with (or without) recombinant human Pdgf (100ng/ml), Sparcl1 (10ng/ml), Vegfc (100ng/ml), Fgf1 (100ng/ml), Ptn (10ng/ml), Timp3 (100ng/ml), Tmsb4x (50ng/ml) or Activin A (100 ng/ml) and reduced concentration of Insulin (0.2 µg/ml). Cultures were maintained for up to 25 days in a humidified atmosphere of 5% CO₂ at 37°C.

Mixed primary oligodendrocyte cultures

Mixed primary oligodendrocyte cultures were prepared from (7 pooled) spinal cords of P4 mouse pups. Tissue chunks were incubated in 5 ml papain (200U) in papain buffer plus L-cysteine for 1 hour at ~35°C with constant gassing (95% oxygen and 5% carbon dioxide). The papain reaction was stopped with plating medium (30% horse serum in DMEM) containing 2.5 µg/ml DNase I. Cells were dissociated into a single cell suspension by triturating through 20 and 23 gauge needles (4 and 2 times, respectively), spun at 800 rpm for 5 min, and the pellet was resuspended in plating medium. Cells were plated at a concentration of 80,000 cells/100 µl plating medium onto PLL coated 11 mm diameter glass coverslips, in 35 mm diameter Petri-dishes (2 coverslips/dish). Two hours later, differentiation medium containing DMEM (4,500 mg/L glucose), 10 ng/ml biotin, 0.5% hormone mixture (1 mg/mL apotransferrin, 20

mM putrescine, 4 μ M progesterone, and 6 μ M selenium), 50 nM hydrocortisone, and 10 μ g/ml insulin (all reagents from Sigma) was added to each 35 mm Petri dish to yield a mix of 50% plating medium: 50% differentiation medium. The following day all medium and myelin debris was removed from the coverslips by rinsing briefly in medium, and fresh differentiation medium was added to each 35 mm Petri dish, with or without human recombinant forms of Klotho (400ng/ml), Ptn (10ng/ml), Timp3 (100ng/ml) or Activin A (100ng/ml). Cells were fed every second day by replacing half the medium with fresh differentiation medium including the recombinant proteins. Cultures were maintained for 3 and 5 days in a humidified atmosphere of 5% CO₂ at 37°C.

Recombinant proteins

Human recombinant proteins were Sparcl1, Vegfc, Klotho, Timp3, and Activin A (R&D Systems), Pdgf and Ptn (Sigma Aldrich), Tmsb4x (Novus Biologicals) and Fgf1 (Invitrogen). Recombinant proteins were dissolved in ddH₂O. Individual aliquots were thawed only once and diluted in cell culture medium immediately before use. PI3K inhibitor LY294002 and MEK1/2 inhibitor UO126 were from Cell Signaling Technology, SMAD3 inhibitor SIS3 was from Calbiochem.

Immunocytochemistry

Cells were fixed with 4% PFA for 5 min at 37°C. After fixation, cultures were washed in PBS three times, permeabilized with ice cold methanol for 5 min, and blocked in blocking buffer (2% fish skin gelatin, 2% FCS, 2% BSA in PBS) for 60 min. Primary antibodies (diluted in 10% blocking buffer) were directed against GFAP (1:200, Novocastra, #NCL-GFAP-GA5), KI-67 (1:10, Dako, #M7249), MBP (1:200, Covance, #SMI-94R), Olig2 (1:200, kindly provided by J. Alberta, PMID: 15198128) SMI31 (1:1000, Covance, #SMI-31P), and APC-CC1 (1:100, Calbiochem, #OP80) and incubated overnight at 4°C. Coverslips were washed in PBS three times. Corresponding secondary Alexa Fluor 488-, 555- and 633-conjugated antibodies (1:1000, Thermo Fisher Scientific, #A-28175, #A-21422, #A-27039 and #A-21094) were diluted in 10% blocking buffer and added for 1 h. Coverslips were washed in ddH₂O three times and mounted in Aqua-Poly/Mount (Polysciences).

Morphometry

Digitized overlapping light microscopic images (20x) were fused to a continuous image of a complete parasagittal cerebellum (Lateral, 0.12 mm) by using Zeiss Zen software and analyzed for absolute numbers of CNP+, CAII+, Olig2+, Iba1+, Mac-3+, and BrdU+ cells. The number of Iba1+ and Mac-3+ cells was normalized to the area of ML and GL, respectively. Endothelial cells positive for CD31 were traced with ImageJ and normalized to the area of the ML. To quantify MBP+ and GFAP+ areas, two ImageJ plugins for semi-automated analysis were implemented (www1.em.mpg.de/Goebbels2016_MBP, www1.em.mpg.de/Goebbels2016_GFAP). Average diameter of hippocampal CA3 neuronal cell bodies of *Nes-CreERT2*Pten^{loxP/loxP}* mice was determined by using ImageJ software. Two independent sections per mouse and staining were quantified. In cell culture experiments, the differentiation and proliferation state of oligodendrocyte lineage cells was analyzed within the given fields of 11 randomly taken images (20x) per coverslip (mixed myelinating cultures: myelination assay, 3-11 independent experiments with 2 coverslips per condition; proliferation assay, 3-11 independent experiments with 1 coverslip per condition; mixed primary oligodendrocyte cultures: 3-5 independent experiments with 2 coverslips per condition). For quantitative

assessment of *in vitro* myelination a 'myelination index' was calculated by dividing the MBP+ area (as identified by generating a 'mask' outlining the myelin sheaths; ImageJ plugin: www1.em.mpg.de/Goebbels2016_CoCultureMBP) by the axonal area (Smi31+ axons; ImageJ plugin: http://www1.em.mpg.de/Goebbels2016_CoCultureSmi31). For the proliferation assay the percentage of semi-automatically quantified Olig2+ cells was determined in relation to all DAPI+ cells (Olig2: ImageJ plugin: http://www1.em.mpg.de/Goebbels2016_CoCultureOlig2; Dapi: ImageJ plugin: http://www1.em.mpg.de/Goebbels2016_CoCultureDapi) and the number of Ki67+, Olig2+ cells was set in relation to the total number of Olig2+ cells. For the differentiation assay, the percentage of postmitotic oligodendrocytes was determined by quantifying and dividing the numbers of CC1+ cells by the total number of Olig2+ oligodendrocyte lineage cells. All analyses were performed in a single blinded fashion towards the investigator who was unaware of the treatment regimen.

G-ratio measurement

Digitized images (magnification 7000x) of ultrathin cerebellum lobe 5 sections were used to determine the numbers of myelinated axons, axon calibers and g-ratios (>100 randomly chosen myelinated axons in the ML). g-ratios were determined by dividing axon diameter by the diameter of the entire fiber (in Fig.1k) or by dividing the inner myelin diameter by the diameter of the entire fiber (in Fig.3g and Supplementary Fig.7c). Quantitations were performed from ≥3 age-matched male mice per genotype and age.

Protein Analysis

Cerebellum lysates were homogenized in lysis-buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 5 mM EGTA, 10% glycerol, 1% triton X-100, 2mM Na₃VO₄) containing phosphatase (PhosSTOP Inhibitor Cocktail, Roche, Basel, Switzerland) and protease (Complete tablets, Roche, Basel, Switzerland) inhibitors using an Ultraturrax (T8, Ika, Staufen, Germany) at highest settings (30–60 s). After incubation for 15 min on ice insoluble material was removed by centrifugation at 16.000 g, 4°C for 15 min. For Westernblot analysis, 10 µg of protein lysate was size-separated on 12% of SDS-polyacrylamide gels and blotted onto PVDF membranes (HybondTM-P, Amersham Biosciences, UK) by Bio-Rad western blotting method. Membranes were blocked in 5% milk powder (in PBS) for 1h at room temperature. Primary antibodies were directed against p-AKT1 (Cell Signaling, mRb, 1:1000, #4060), AKT1 (Cell Signaling, mRb, 1:1000, #4691), PTEN (Cell Signaling, mRb, 1:1000, #9188), p-GSK3β (Cell Signaling, mRb, 1:1000, #9336), p-mTOR (Cell Signaling, mRb, 1:1000, #5536), p-S6 (Cell Signaling, mRb, 1:1000, #4858), and Actin (Sigma–Aldrich, mM, 1:1000, #A3853). Antibodies were diluted in blocking buffer and incubated overnight at 4°C. Membranes were washed 3 x 10 min in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% Tween-20), followed by an incubation with horse-radish peroxidase-conjugated secondary antibodies (1:5000, Dianova, #111-035-003, #115-035-003). After three more washing steps with TBS-T buffer, immunoreactive proteins were detected with an enhanced chemiluminescence kit (Western LightningTM, Westernblot Chemiluminescence Reagent Plus, PerkinElmer Life Sciences, Waltham, MA) according to the manufacturer's instructions.

Statistical analysis

For Power analysis the software G*Power Version 3.1.7. was used. Power analyses were performed before conducting experiments (a priori). Adequate Power (1 – beta-error) was defined as $\geq 80\%$ and the alpha error as 5%. The sample size was calculated with the following pre-specified effect sizes: 1. mRNA expression analysis: effect size d of approximately 2.5 (estimated mean difference of 50% and standard deviation of 15%). 2. Analysis of mature oligodendrocyte numbers in GL (effect size d of approximately 3.5, estimated mean difference of 30% and standard deviation of 10%) and ML (effect size d of approximately 6.5, estimated mean difference of 400% and standard deviation of 30%) and myelinated area in the ML of wildtype and *Pten* mutants (effect size d of approximately 6.0, estimated mean difference of 170% and standard deviation of 40%). 3. Proliferation analysis of oligodendrocyte lineage cells: effect size d of approximately 9.0 (estimated mean difference of 330% and standard deviation of 15%). 4. *In vivo* analysis of candidate genes. Analysis of mature oligodendrocyte numbers: effect size d of approximately 5.5 (estimated mean difference of 30% and standard deviation of 15%). Analysis of myelinated area: effect size d of approximately 4.0 (estimated mean difference of 30% and standard deviation of 13%). Proliferation analysis of oligodendrocyte lineage cells: effect size d of approximately 3.5 (estimated mean difference of 20% and standard deviation of 10%). Analysis of (i) myelinated and (ii) unmyelinated pf diameters: (i) effect size d of approximately 2.6 (estimated mean difference of 20% and standard deviation of 13%). (ii) Effect size d of approximately 1.5 (estimated mean difference of 15% and standard deviation of 13%). G-ratio analysis: effect size d of approximately 4.5 (estimated mean difference of 6% and standard deviation of 2%). 5. Microglia (ML: Effect size d of approximately 4.7, estimated mean difference of 100% and standard deviation of 20%; GL: effect size d of approximately 1.2, estimated mean difference of 10% and standard deviation of 5%), astrocytes (ML: effect size d of approximately 4.0, estimated mean difference of 150% and standard deviation of 15%; GL: effect size d of approximately 1.3, estimated mean difference of 10% and standard deviation of 5%) and vascular endothelia cells (effect size d of approximately 3.7, estimated mean difference of 40% and standard deviation of 15%) in *Pten* mutant mice. 6. Microglia (ML: effect size d of approximately 1.0, estimated mean difference of 10% and standard deviation of 9%; GL: effect size d of approximately 1.3, estimated mean difference of 10% and standard deviation of 7%) and astrocytes (ML: effect size d of approximately 1.2, estimated mean difference of 20% and standard deviation of 15%; GL: effect size d of approximately 1.1, estimated mean difference of 10% and standard deviation of 5%) in *NEX-CreERT2*Pten^{loxP/loxP}* mice. 7. Average diameter of hippocampal CA3 neuronal cell bodies from *Nex-CreERT2*Pten^{loxP/loxP}* mice: effect size d of approximately 12.0 (estimated mean difference of 25% and standard deviation of 2%) 8. *In vitro* analysis of candidate factors. Analysis of oligodendrocyte lineage cell numbers: effect size d of approximately 4.0 (estimated mean difference of 80% and standard deviation of 30%). Proliferation analysis: effect size d of approximately 2.3 (estimated mean difference of 60% and standard deviation of 36%). Myelination index: effect size d of approximately 2.0 (estimated mean difference of 65% and standard deviation of 50%). Analysis of oligodendrocyte differentiation: effect size d of approximately 9.0 (estimated mean difference of 40% and standard deviation of 10%). For electrophysiology experiments we determined the sample size by utilizing previous data distributions.

Data are expressed as means $\pm$ s.e.m. In order to select appropriate statistical tests all data have been tested for normal distribution with Kolmogorov-Smirnov test or

Shapiro-Wilk test. For normally distributed data with comparable variances we used two-tailed unpaired Student's t-tests and Chi-square tests to determine the statistical significance between two groups. For data not showing normal distribution or in case that no normality test could be conducted the nonparametric Wilcoxon matched pairs test was applied. Data sets containing more than two groups were tested by applying analysis of variance (ANOVA) and Bonferroni post hoc test. Analysis of co-variance (ANCOVA) was used to analyze the differences between slopes of regression lines. Applied statistical tests are indicated in the respective figure legends. Statistical differences were considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and tests and fitting of regression lines were performed by using GraphPad (Prism) and MS Excel.

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Fig.1

Fig.2

Fig.3