Microglial neuropilin-1 promotes oligodendrocyte expansion during development and remyelination by trans-activating platelet-derived growth factor receptor

Amin Sherafat1, Friederike Pfeiffer1,2, Alexander M. Reiss1, William M. Wood1 & Akiko Nishiyama1,3,4

Nerve-glia (NG2) glia or oligodendrocyte precursor cells (OPCs) are distributed throughout the gray and white matter and generate myelinating cells. OPCs in white matter proliferate more than those in gray matter in response to platelet-derived growth factor AA (PDGF AA), despite similar levels of its alpha receptor (PDGFRα) on their surface. Here we show that the type 1 integral membrane protein neuropilin-1 (Nrp1) is expressed not on OPCs but on amoeboid and activated microglia in white but not gray matter in an age- and activity-dependent manner. Microglia-specific deletion of Nrp1 compromised developmental OPC proliferation in white matter as well as OPC expansion and subsequent myelin repair after acute demyelination. Exogenous Nrp1 increased PDGF AA-induced OPC proliferation and PDGFRα phosphorylation on dissociated OPCs, most prominently in the presence of sub-optimum concentrations of PDGF AA. These findings uncover a mechanism of regulating oligodendrocyte lineage cell density that involves trans-activation of PDGFRα on OPCs via Nrp1 expressed by adjacent microglia.
Oligodendrocyte precursor cells (OPCs), also known as NG2 glia or polydendrocytes, are widely distributed throughout the developing and mature central nervous system (CNS)\(^1-3\), where they comprise 2–9% of the total cells. The developmental expansion of this population is critically dependent on platelet-derived growth factor AA (PDGF AA) acting on their alpha receptor (PDGFR\(\alpha\))\(^4-6\). The most characterized and established role of OPCs is their ability to self-renew and generate the correct number of myelinating oligodendrocytes (OLs) needed for optimal network function. Hypomyelinating or dysmyelinating mouse mutants with reduced OLs or myelin are accompanied by increased OPC proliferation\(^7,8\). In the mature CNS, a demyelinated lesion rapidly elicits OPC proliferation\(^9-13\). However, the molecular and cellular mechanism by which such feedback signals promote OPC proliferation is not known.

While OPCs are evenly distributed throughout the CNS, the rate of OPC proliferation and OL differentiation is greater in white matter than in gray matter\(^13,14\), and those in white matter proliferate more in response to PDGF AA\(^15\). While both cell intrinsic and extrinsic factors influence the differential OPC behavior\(^16\), heterotypic transplantation of 300-μm² pieces in slice cultures suggests that the greater proliferative response of OPCs in white matter to PDGF AA is imparted by their local pericellular microenvironment\(^15\).

To determine the mechanism underlying the differential response of OPCs in gray and white matter to PDGF AA, we searched the literature for a potential co-receptor for PDGF that could differentially regulate the proliferative response of OPCs to PDGF AA in gray and white matter. We identified heparan sulfate proteoglycans (HSPGs), and neuropilin-1 (Nrp1)\(^17,18\) as potential candidates. Since heparin and HSPGs are known to affect a variety of growth factors, we chose to explore the role of Nrp1 as a molecule with more specific targets. Nrp1 is a type 1 transmembrane molecule, which was first discovered in the developing *Xenopus* optic tectum\(^19\) and subsequently shown to be expressed on developing murine axons\(^20\) and regulate axon pathfinding by binding to class III semaphorins\(^21\). In endothelial cells, Nrp1 binds vascular endothelial cell growth factor (VEGF) and modulates signal transduction through VEGF receptor (VEGFR)\(^22\). Here, we show that Nrp1 is expressed on ameboid and activated microglia in the developing and demyelinated corpus callosum and promotes OPC proliferation by activating PDGFRa on OPCs in trans.

### Results

**Nrp1 modulates PDGF AA-dependent OPC proliferation in white matter.** To determine whether the proliferative response of OPCs to PDGF AA was modulated by Nrp1, we tested the effects of anti-Nrp1 antibody on PDGF AA-induced OPC proliferation in forebrain slice cultures from postnatal day 8 (P8) NG2creZ/EY mouse double transgenic mice\(^23\) (Fig. 1A). In the presence of 50 ng/mL PDGF AA alone, 42% of EYFP+ OPCs in the corpus callosum were EdU+ (Fig. 1D, F). By contrast only 18.4% of those in the cortex were EdU+ in the presence of PDGF AA (Fig. 1B, F). Anti-Nrp1 antibodies elicited a dose-dependent decrease in the proportion of EYFP+ cells that proliferated in response to PDGF AA in the corpus callosum (Fig. 1F), but there was no effect on basal OPC proliferation in gray matter (Fig. 1C, F). We did not observe any increase in TUNEL+ cells in the anti-Nrp1 antibody-treated slices (Fig. 1G, H) compared to slices treated with a control IgG, indicating that the antibody did not cause significant cell death. Thus, Nrp1 appeared to be necessary for PDGF AA-mediated OPC proliferation in the corpus callosum.

**Nrp1 is expressed on ameboid microglia in the developing corpus callosum.** To determine the relevant source of Nrp1 that could modulate PDGF AA-dependent OPC proliferation in the corpus callosum, we examined the developing mouse CNS tissues for Nrp1 expression. In P5 brain, Nrp1 was detected widely in the forebrain (Fig. 2A), including vascular cells (arrowheads in Fig. 2A, C) along laminin+ blood vessels (Fig. S1A), as expected from previous reports on Nrp1 on vascular endothelial cells\(^22\). Nrp1 immunoreactivity was also detected on axons in the dorsal spinal cord (Fig. S1B) in P5 mice and in the dorsal corpus callosum in E18.5 brain (Fig. S1C), as previously shown (Kawakami et al. 1996). In addition, there was strong cellular staining in a subregion of the corpus callosum (Fig. 2A, boxed area magnified in Fig. 2D–F). The majority of the round Nrp1+ cells in the corpus callosum also expressed the microglial cell surface antigen F4/80\(^23\) and had the appearance of ameboid microglia (Fig. 2A–F). Similarly, we detected Nrp1 on a subpopulation of F4/80+ ameboid microglia in P5 cerebellar white matter (not shown). We did not detect Nrp1 on PDGFRa+ OPCs (Fig. 2G–I), although Nrp1+ microglial processes were closely apposed to OPC cell bodies and processes (arrows in Fig. 2I). The close proximity to Nrp1-expressing ameboid microglia to OPCs suggested that microglial Nrp1 in the white matter could be modulating the proliferative response of OPCs to PDGF AA.

To further examine the enrichment of Nrp1 among microglia in white matter, we examined the expression of Nrp1 on EYFP+ cells in the neocortex and corpus callosum of Cx3CR1creERT2-ires-EYFP mice from P5 to P30 (Fig. 2J–M and Fig. S2). At P5 and P8, Nrp1 was expressed on the majority of EYFP+ ameboid microglia in the corpus callosum (97.8% at P5 and 98.4% at P8), but the enrichment declined by P14 to 14.0% (Fig. 2M), and Nrp1 was no longer detected at P30, as the EYFP+ microglia in the corpus callosum displayed progressively more ramified morphology. In the cortex, EYFP+ microglia had ramified morphology from P5 through P30 and did not co-express Nrp1. Thus, Nrp1 was preferentially expressed on ameboid cells in the early postnatal white matter.

The ameboid microglia that were found in P5 corpus callosum also expressed CD68 (Fig. S3, A–D, arrows), which is a lysosomal marker\(^24,25\) and hence an indicator of phagocytic activity. The microglia in P14 cortex callosum exhibited a very small amount of punctate immunoreactivity for CD68, which was significantly lower compared to that in P5 corpus callosum, and these microglia were Nrp1-negative (Fig. S3, E–H, arrowheads).

**Microglia-specific Nrp1 deletion reduces developmental OPC proliferation in white matter.** To examine the effects of microglial Nrp1 on OPC proliferation, we deleted Nrp1 specifically from microglia using Nrp1 conditional knockout mice\(^26\) crossed to Cx3CR1creERT2-ires-EYFP mice, which express tamoxifen-inducible cre specifically in Cx3CR1-expressing microglia\(^27\) (Fig. 3A). When we induced Nrp1 deletion in microglia in mg-Nrp1-cont (Il/+ and mg-Nrp1-cko (Il/Il) mice by injecting 4-hydroxytamoxifen (4-OHT) at P2 and P3 and analyzed at P5, none of the EYFP+ microglia in mg-Nrp1 cko mice had detectable Nrp1 expression in the corpus callosum, whereas Nrp1 was detected on >99% of EYFP+ microglia in mg-Nrp1 cont heterozygous mice (Fig. S4A–D). Microglial Nrp1 deletion did not affect the density of microglia or astrocytes (Fig. S4E, F). Nrp1 immunoreactivity on blood vessels remained detectable in mg-Nrp1-cko (Fig. S4C, arrow), and the relative area in the corpus callosum occupied by blood vessels was not altered in mg-Nrp1-cko (Fig. S4G).

Having confirmed microglia-specific deletion of Nrp1 with this model, we next examined OPC proliferation in the corpus callosum of mg-Nrp1-cont and cko mice by EdU pulse labeling at P5 after microglial Nrp1 deletion at P2 and P3 (Fig. 3B). Comparison of the density of PDGFRa+ EdU+ cells in mg-
Nrp1-cont and cko corpus callosum revealed a 2.6-fold reduction in cko (Fig. 3C, D, E). Since we observed that OPCs were closely apposed to Nrp1+ microglia in P5 corpus callosum as described above (Fig. 2G–I), we investigated whether OPCs that were in contact with Nrp1+ microglia were proliferating more than OPCs without microglial contact. We used 3D rotation of series of ≥40 confocal z-slices from P5 sections labeled for PDGFRα, Nrp1, EYFP, and EdU and assessed whether each PDGFRα+ OPC had an EYFP+ microglial element directly apposed to it and whether it had incorporated EdU (Fig. 3C, D, arrows; Fig. S5). Among the proliferating OPCs, 95.9% in mg-Nrp1-cont and 92.8% in mg-Nrp1-cko had contact with microglia. In mg-Nrp1-cont corpus callosum, the contacts often involved intertwining processes of a PDGFRα+ OPC with microglia that expressed Nrp1 primarily on their processes. When we saw a direct apposition of microglia and OPC by confocal microscopy, we scored the OPC as having contact (Fig. S5A–G). When we did not detect direct apposition through all the z-stacks we scored them as having no contact (Fig. S5H–N). There was no significant difference in the density of proliferating OPCs that were not in contact with microglia between the two genotypes (Fig. 3E, triangles). By contrast, the density of proliferating OPCs that were in contact with microglia was 2.7-fold lower in mg-Nrp1-cko compared to cont (Fig. 3E, circles). This indicated that the

Fig. 1 Anti-Nrp1 antibody blocks PDGF AA-mediated OPC proliferation in white but not gray matter. A Schematic showing slice cultures from P8 NG2cre:Z/EG mice to assay for OPC proliferation in response to 50 ng/mL PDGF AA and different concentrations of anti-Nrp1 antibody. B–E Slice cultures from P8 NG2cre:Z/EG mice that were fixed and labeled for EGFP (green) and EdU (magenta) in the cortex (B and C) or corpus callosum (D and E) in the presence of 50 ng/mL PDGF AA in the absence (B and D) or presence (C and E) of 1 µg/mL anti-Nrp1 antibody. Arrows, examples of EGFP+ EdU+ cells. Scale bars, 50 µm. F Quantification of OPC proliferation in slice cultures after 5 h of EdU labeling. y-axis, proportion of EGFP+ cells that were EdU+. Tukey’s multiple comparisons test, n = 4, F(4, 30) = 9.966, means ± standard deviations. The differences between OPC proliferation in gray and white matter were significant at anti-Nrp1 antibody concentrations of 0 (p < 0.001) and 0.01 µg/mL (p = 0.0065), but not at higher concentrations of the antibody. Black, gray matter; gray, white matter. Two-way ANOVA, Tukey’s multiple comparisons test, F(1, 30) = 89.71, n = 4. G, H Slice cultures treated with 1 µg/mL control goat IgG (G) or goat anti-Nrp1 antibody (H) and labeled for EGFP (green) and TUNEL (magenta). Arrows indicate a TUNEL+ cell. Scale bars, 50 µm. Source data are provided as a Source Data file.
Fig. 2 Nrp1 expression in the developing postnatal brain. A–C Low-magnification view of a coronal forebrain section labeled for Nrp1 (red) and F4/80 (green). Arrowheads, examples of Nrp1+ blood vessels. Arrowheads, blood vessels. Boxed area with a cluster of Nrp1+ cells is shown in D–F. Scale, 100 µm. ctx, cortex; cc, corpus callosum; LV lateral ventricle. D–F High-magnification view showing Nrp1+ cells in the corpus callosum express F4/80. Arrows in F indicate blood vessels that are F4/80-negative. Scale, 50 µm. G–I Double labeling for Nrp1 (red) and PDGFRα in P5 corpus callosum. Nrp1 is not expressed on PDGFRα+ OPCs, but Nrp1+ processes are found close to OPC cell bodies and processes (arrows in I). J–L Double labeling for Nrp1 (red) and YFP (green) in Cx3CR1creERT2-ires-EYFP mice from P5 through P14. Scale = 50 µm. M The proportion of EYFP+ microglia that expressed Nrp1 in the corpus callosum. One-way ANOVA, Tukey’s multiple comparisons test, n = 3, F(2, 6) = 438.8. Source data are provided as a Source Data file.
decrease in OPC proliferation in the corpus callosum of P5 mg-Nrp1-cko was largely due to the decrease in OPCs that were in contact with microglia. In mg-Nrp1-cko, the proportion of total PDGFRα+ OPCs that were contacting microglia, regardless of EdU incorporation, was 22% lower than that in mg-Nrp1-cont (Fig. 3F), suggesting that the presence of Nrp1 on microglia could be facilitating OPC contact.

We next examined whether the decrease in OPC proliferation in the white matter of mg-Nrp1-cko mice was restricted to the period in which Nrp1 was highly expressed on ameboid microglia. While the proportion of NG2+ OPCs that had incorporated EdU+ was significantly lower in the cerebellar white matter and corpus callosum of P5 mg-Nrp1-cko, the difference was no longer seen in P14 corpus callosum (Fig. 3G).
Thus, the developmental age during which microglial Nrp1 deletion reduced OPC proliferation coincided with the temporal window during which Nrp1 was expressed on ameboid microglia in the corpus callosum (Fig. 2M, Fig. S2).

The density of total OPCs in the corpus callosum of mg-Nrp1-cko mice was 1.7-fold lower than that in control mice at P5, but by P14 the difference was no longer detected (Fig. 3H). TUNEL labeling did not reveal any TUNEL+ cells in the corpus callosum of mg-Nrp1-cont or mg-Nrp1-cko at P5 (Fig. S4H–J), suggesting that microglial deletion of Nrp1 did not increase cell death. The transient reduction in OPC density did not lead to long-lasting changes in myelination, as judged by comparable levels of myelin basic protein (MBP) immunofluorescence intensity in the corpus callosum in P14 mg-Nrp1-cont and cko mice (Fig. 3I and Fig. S4K–P).

Microglia-specific Nrp1 deletion reduces PDGF AA-mediated OPC proliferation in slice culture. To examine whether microglial deletion of Nrp1 compromised the proliferative response of OPCs to PDGF AA, we prepared slice cultures from P8 mg-Nrp1-cont and mg-Nrp1-cko mouse forebrains after 4-OHT administration at P2-3 and examined the proliferation of OPCs in gray and white matter in response to 50 ng/mL PDGF AA (Fig. S6A). There was no effect of Nrp1 deletion on EdU incorporation into gray matter OPCs. By contrast, EdU incorporation into OPCs in the corpus callosum of mg-Nrp1-cko slices was reduced to 57% of mg-Nrp1-cont.

To further examine whether Nrp1-deleted microglia directly affected OPC proliferation, we cocultured OPCs with perinatal microglia from mg-Nrp1-cont and mg-Nrp1-cko forebrain and assayed for OPC proliferation in the absence or presence of 15 ng/mL PDGF AA (Fig. S6B). OPC proliferation was significantly lower when cocultured with mg-Nrp1-cko microglia compared with mg-Nrp1-cont microglia. In the presence of a non-saturating concentration (15 ng/mL) of PDGF AA, the significant increase in OPC proliferation observed in OPCs cocultured with mg-Nrp1-cont microglia was not observed in OPCs cocultured with mg-Nrp1-cko microglia. Thus, the presence of Nrp1 on microglia plays a significant role in promoting OPC proliferation. The compromised OPC proliferation observed with cko microglia even in the absence of exogenous PDGF AA could reflect either PDGF AA secretion from microglia or OL lineage cells present in the culture or other PDGF AA-independent effects of microglia on OPC proliferation.

Microglial Nrp1 deletion compromises OPC expansion after demyelination. Our immunolocalization studies revealed that Nrp1 was abundantly expressed on ameboid microglia that appeared transiently in the corpus callosum during the first postnatal week and was downregulated on microglia by P14 as they became more ramified. To determine whether Nrp1 expression could be upregulated in pathologic conditions that are known to increase OPC proliferation, we used an acute chemically induced demyelination model created by injecting a-lysophosphatidylcholine (LPC, lysolecithin) into the corpus callosum of 2- to 3-month-old mg-Nrp1-cont and mg-Nrp1-cko mice (Fig. 4A). Nrp1 was deleted in microglia by tamoxifen injection prior to induction of demyelination, with a deletion efficiency of 99.5% of YFP+ cells. We first examined whether Nrp1 was re-expressed on microglia after demyelination in mg-Nrp1-cont mice. Three days after PBS injection, YFP+ cells surrounding the injection site had the morphology of resting ramified microglia and did not express Nrp1 (Fig. 4B). By contrast, 3 days after LPC injection (3 dpl), there was strong activation of microglia, and Nrp1 was robustly upregulated on the activated YFP+ microglia/macrophages (Fig. 4C). At 7 dpl, YFP+-activated microglia/macrophages were abundantly detected and expressed the lysosomal protein CD68 in the demyelinated lesion of both mg-Nrp1-cont and mg-Nrp1-cko corpus callosum (Fig. 4D, E). In mg-Nrp1-cont lesions, Nrp1 immunoreactivity was detected on the surface of CD68+ macrophages (Fig. 4D, arrows). This indicated that Nrp1 was highly upregulated on activated microglia/macrophages that appeared in the demyelinated corpus callosum. In the cko lesions, the majority of the CD68+ cells lacked Nrp1 (Fig. 4E, arrowheads). While Nrp1 was undetectable in 99.5% of YFP+ cells at 3 dpl, we detected a small number of CD68+ YFP- cells that expressed Nrp1 in the cko corpus callosum at 7 dpl (Fig. 4E, arrows), which were likely to have entered the lesion from Cx3cr1-negative precursors. The density of YFP+ microglia/macrophages was similar in mg-Nrp1-cont and mg-Nrp1-cko mice at 3 dpl (Fig. 4F), indicating that the lack of Nrp1 on microglia in mg-Nrp1-cko lesions did not affect their infiltration or activation.

At 3 and 7 dpl, we pulse-labeled LPC-injected mice with EdU and examined the effect of microglial Nrp1 deletion on OPC proliferation in the lesion. EdU incorporation into YFP-negative, NG2+ OPCs at the lesion site in mg-Nrp1-cko was 1.9-fold lower than that in mg-Nrp1-cont lesions at 3 dpl and 3.2-fold lower at 7 dpl (Fig. 4F–K). OPC proliferation was 2- to 3-fold higher at 3 dpl compared to 7 dpl for both genotypes. The time course of OPC proliferation is consistent with our previous report of an early proliferative response in the region immediately surrounding the core demyelinated lesion. These observations indicate that loss of Nrp1 on activated microglia significantly compromised the early proliferative response of OPCs to acute demyelination.

Microglial Nrp1 deletion compromises OL regeneration and myelin repair. To examine whether the reduced proliferation of OPCs in mg-Nrp1-cko mice affected subsequent events in myelin regeneration, we examined the lesion at 14 dpl. Quantification of OLs in and around the lesion at 14 dpl revealed that the density of CC1+ OLs in mg-Nrp1-cko lesion was reduced to 63% of that in
mg-Nrp1-cont lesion (Fig. 5A–C). To assess the extent of myelin repair, we first estimated the demyelinated area at 14 dpl by taking the area that exhibited reduced myelin basic protein (MBP) and elevated non-phosphorylated neurofilament immunoreactivity. The demyelinated area at 14 dpl was 1.74 times larger in the mg-Nrp1-cko mice compared with that in the mg-Nrp1-cont mice (Fig. 5D–F). Thus, the reduction of oligodendrocyte density in the knockout animals correlated with the larger area containing demyelinated axons, suggesting that the repair process was impaired in mg-Nrp1-cko mice.

We next examined the impact of Nrp1 deletion from activated microglia on remyelination at 28 dpl. MBP immunofluorescence intensity in the lesioned corpus callosum of mg-Nrp1-cko mice was lower, at 61% of that in mg-Nrp1-cont mice (Fig. 5G–I).
There was a higher level of non-phosphorylated neurofilament in the cko, which is consistent with prolonged impairment of myelin repair. To examine the extent of myelin repair in more detail, we performed ultrastructural analysis of the lesioned corpus callosum of mg-Nrp1-cont and mg-Nrp1-cko mice at 28 dpl (Fig. 5J–K and Fig. S7A–F) and searched for signs of myelin repair process. In mg-Nrp1-cont lesions, we saw that most axons were myelinated (Fig. 5J), and that some axons were surrounded by thin or less compacted myelin sheaths indicating remyelination (arrows in Fig. 5J), while very few axons were found...
unmyelinated (arrowhead in Fig. 5J). By contrast, in thecko
lesions (Fig. 5 K), there were more axons without myelin
(arrowheads in Fig. 5K), and the tissue appeared more
heterogeneous compared to the control and contained
swollen glial processes (asterisks in Fig. 5K). We observed
less compacted myelin sheaths (arrow in Fig. 5K), but overall,
less myelin as compared to mg-Nrp1-cko (Fig. 5J). Some glial
processes were present in the lesions of mg-Nrp1-1-cko mice (Fig.
S7A), but more glial cell processes as well as glial cells
were present in mg-Nrp1-cko mice (Fig. S7D). We detected
an oligodendrocyte in the process of ensheathing an axon (arrow
in Fig. S7B) in the lesion of mg-Nrp1-cont mice, while the
oligodendrocyte in mg-Nrp1-cko lesion was surrounded by
several axons with un-compacted to more compacted myelin
(Fig. S7E). Concomitantly, many axons displayed a glial
ensheathment that was not yet compacted in the lesions of mg-
Nrp1-cko mice (arrows in Fig. S7F), while this was less abundant
in the mg-Nrp1-cont mice (arrow in Fig. S7C). These observations
suggest that the remyelination process in mg-Nrp1-cko mice
was not completely blocked but greatly delayed compared to
the controls. Thus, failure to upregulate Nrp1 on activated
microglia after acute demyelination significantly impaired
the initial proliferative response of OPCs and compromised
the timely production of new oligodendrocytes, causing a significant
delay in the subsequent remyelination process.

Exogenous Nrp1 augments PDGF-dependent OPC prolifera-
tion by augmenting PDGFRα phosphorylation on OPCs. The
above experiments revealed that Nrp1 on microglia is critical for
PDGF AA-mediated proliferation of OPCs in the white matter and
for the proliferative response of OPCs to acute demyelina-
tion. We next examined whether excess Nrp1 could augment the
proliferative response of OPCs. Exogenous Nrp1 was added to
slice cultures from P8 NG2cre;Z/EG mice in the form of soluble
Nrp1-Fc fusion protein. Nrp1-Fc consisted of the extracellular
slice cultures from P8 NG2cre;Z/EG mice in the form of soluble
proliferative response of OPCs. Exogenous Nrp1 was added to
and for the proliferative response of OPCs to acute demyelina-
tion. Scale, 50 µm. LV, lateral ventricle. F. Quantification of demyelinated area in cont and cko lesions at 14 dpl. Student’s t-test,
unpaired, two-tailed, n = 5, t = 4.753, df = 8. G, H. Labeling for MBP (blue) and non-phosphorylated neurofilaments (non-P-NF, red) using smi-32 antibody to identify axons that have not yet undergone complete remyelination. Dotted lines indicate the extent of partial or
complete demyelination. Scale, 50 µm. LV, lateral ventricle. F. Quantification of demyelinated area in cont and cko lesions at 14 dpl. Student’s t-test,
unpaired, two-tailed, n = 5, t = 4.753, df = 8. Black circles, mg-Nrp1-cont; blue squares, mg-Nrp1-cko in C, F, and I. J, K. Electron microscopic images of cross-sections of LPC-lesioned corpus callosum at 28 dpl. J mg-Nrp1-cko (fl/fl) showing sheaths of various thickness (arrows), but most axons appear fully myelinated. K. mg-Nrp1-cko (fl/fl) showing more axons without myelin sheaths (arrowheads), axons with thin myelin
(arrows), and abundant swollen glial cell processes (*). Scale bars 1 µm. Source data are provided as a Source Data file.

The extent of oligodendrocyte regeneration and myelin repair in mg-Nrp1-cko. A, B Labeling mg-Nrp1-cont (L) and cko (M) for CC1 (red), DAPI (blue), and EYFP (green) at 14 dpl. Arrowheads, CC1+ cells. Scale, 25 µm. C Quantification of OL density in cont and cko lesions at 14 dpl. Student’s t-test,
unpaired, two-tailed, n = 5, t = 4.753, df = 8. D, E Labeling mg-Nrp1-cont (O) and cko (P) for MBP (blue) and non-phosphorylated neurofilaments (non-P-NF, red) using smi-32 antibody to identify axons that have not yet undergone complete remyelination. Dotted lines indicate the extent of partial or
complete demyelination. Scale, 50 µm. LV, lateral ventricle. F. Quantification of demyelinated area in cont and cko lesions at 14 dpl. Student’s t-test,
unpaired, two-tailed, n = 5, t = 4.753, df = 8. G, H. Labeling for MBP (blue) and non-phosphorylated neurofilaments (non-P-NF, red) in the lesioned corpus callosum of mg-Nrp1-cont (G) and cko (H) mice at 28 dpl. I Quantification of MBP immunofluorescence in the lesioned corpus callosum of mg-Nrp1-cont and cko mice at 28 dpl. Student’s t-test, unpaired, two-tailed, n = 5, t = 4.031, df = 8. Black circles, mg-Nrp1-cont; blue squares, mg-Nrp1-cko in C, F, and I. J, K. Electron microscopic images of cross-sections of LPC-lesioned corpus callosum at 28 dpl. J mg-Nrp1-cko (fl/fl) showing sheaths of various thickness (arrows), but most axons appear fully myelinated. K. mg-Nrp1-cko (fl/fl) showing more axons without myelin sheaths (arrowheads), axons with thin myelin
(arrows), and abundant swollen glial cell processes (*). Scale bars 1 µm. Source data are provided as a Source Data file.

Since slice cultures contained different cell types, the above effects of Nrp1-Fc on OPCs could have been mediated by direct effects of Nrp1-Fc on OPCs or by indirectly altering signaling pathways in
microglia or other cell types. To resolve this, we added Nrp1-Fc to
purified dissociated OPC cultures and examined their proliferation
in response to PDGF AA. OPCs were immunopanned from P2-4
mouse neocortex, and 2 µg/mL Nrp1-Fc was added together with 0
to 50 ng/mL PDGF AA (Fig. 7A–E). Immunopanning yielded an
enriched population of OPCs containing <2% F4/80+ microglia.
In the presence of PDGF AA concentrations of 4 µg/mL or lower,
Nrp1-Fc had no effect on OPC proliferation, and fewer than 4% of
Olig2+ OPCs were EdU+ (Fig. 7A, B, E). In the presence of 50 µg/
ML PDGF AA, 54.6% of Olig2+ cells had incorporated EdU
without Nrp1-Fc, and this was further increased to 75.9% by the
addition of Nrp1-Fc. The most prominent effect of Nrp1-Fc was seen in the presence of 15 µg/mL of PDGF AA, increasing EdU
incorporation 3.7-fold from 9% to 33.7% (Fig. 7C–E). These findings indicate that Nrp1 acted directly on OPCs to augment
PDGF AA-induced proliferation, and it was most effective at
suboptimum concentrations of PDGF AA.

Next, we determined whether Nrp1-Fc could augment PDGFRα activation on OPCs exposed to limited amounts of PDGF AA. PDGFRα activation was assessed by immunoblotting for tyrosine
phosphorylation on PDGFRα after treating OPCs for 30 min at 37°C
with 15 µg/mL PDGF AA with or without 2 µg/mL Nrp1-Fc. Immunoblots of protein extracts from the treated cells were
incubated with rabbit and goat antibodies that recognized phos-
phorylated PDGFRα and total PDGFRα, respectively. We detected
a significant increase in the level of phosphorylated PDGFRα in Nrp1-
Fc-treated cells compared with those treated with PDGF AA alone
(Fig. 7F, G). This indicated that Nrp1-Fc could augment the ability
of PDGF AA to phosphorylate PDGFRα on OPCs.

To further examine the interaction between Nrp1-Fc and
PDGFRα on OPCs, we performed co-clustering experiments by
incubating dissociated OPCs with control-Fc or Nrp1-Fc for 30
min at 4°C or 36°C and stained for Fc and PDGFRα, as well as
Olig2. After incubating OPCs with control-Fc consisting of
human IgG Fc dimer at 4°C or 36°C or with Nrp1-Fc at 4°C (Fig.
S8A–C), PDGFRα was found in small puncta along the processes
and some at the soma (A, B), and there was little detectable
distinct Fc immunoreactivity. Incubation of OPCs with Nrp1-Fc
at 36°C caused greater aggregation of PDGFRα, and Fc
immunoreactivity was also found co-clustered with many of the
PDGFRα+ aggregates (Fig. S8D, arrows). These observations
further suggest that Nrp1-Fc binds to and causes functional clustering of PDGFRα.

Discussion

We have shown that Nrp1 expressed by activated microglia plays a critical role in promoting OPC proliferation in early postnatal white matter tracts and after demyelination in the adult corpus callosum. Nrp1 was detected on the majority of ameboid microglia that appeared transiently in the early postnatal white matter, was not detected on resting ramified microglia, and was re-expressed on activated microglia/macrophages after acute demyelination. During both development and after demyelination, Nrp1+ microglia were closely apposed to OPC processes. In slice culture, deletion of Nrp1 in microglia significantly reduced PDGF AA-induced OPC proliferation in white but not gray matter. In vivo, microglial Nrp1 deletion significantly reduced OPC proliferation, and the majority of OPCs that were proliferating in P5 corpus callosum were in contact with microglia. Microglial deletion of Nrp1 had no effect in regions where microglia were ramified and did not express Nrp1, such as the cortex and the mature corpus callosum. In adult corpus callosum, Nrp1 was robustly induced on activated microglia/macrophages after acute demyelination, and loss of Nrp1 in these cells

Fig. 6 Effects of Nrp1-Fc on OPC proliferation in slice cultures from P8 NG2cre;Z/EG mice. A–F Labeling for EGFP (green) and EdU (magenta) in the cortex (A–C) and corpus callosum (D–F) of slices treated with 50 ng/ml PDGF AA only (A, D), 50 ng/ml PDGF and 2 μg/ml Nrp1-Fc (B, E), or 50 ng/ml PDGF AA, 2 μg/ml Nrp1-Fc, and 1 μg/ml goat anti-mouse PDGFRα function-blocking antibody (C, F). Scale, 50 μm. G Dose-response of OPC proliferation in the cortex and corpus callosum to exogenous Nrp1-Fc in the presence of 50 ng/ml PDGF AA. Values are the percentages of EGFP+ cells that were EdU+. Two-way ANOVA, Tukey’s multiple comparisons test, n = 3, F(4, 20) = 5.622 for comparisons among different Nrp1-Fc concentrations and F(4, 20) = 317.6 for gray versus white matter comparison. Black, cortex; gray, corpus callosum. H The effects of anti-PDGFRα blocking antibody on PDGF AA-mediated OPC proliferation in the cortex (gray bars) and corpus callosum (white bars) in the presence (checkered bars) or absence (solid bars) of exogenous Nrp1-Fc. All samples were treated with 50 ng/ml PDGF AA. Two-way ANOVA, Sidak’s multiple comparisons test, n = 4, F(3, 16) = 266.6. Circles, no blocking antibody; squares, PDGFRα blocking antibody. Black and gray, no Nrp1-Fc; blue, 2 μg/ml Nrp1-Fc. Source data are provided as a Source Data file.
significantly reduced OPC proliferation and subsequent oligodendrocyte differentiation and remyelination. Exogenous Nrp1 augmented PDGF AA-induced proliferation of cultured OPCs by increasing phosphorylation and clustering of PDGFRα. These findings support the model in which Nrp1 on microglia modulates PDGFRα-mediated proliferation of adjacent OPCs in trans (Fig. 8).

Dynamic expression of Nrp1 on a subset of “activated” microglia in white matter. CNS parenchymal microglia originate from the embryonic yolk sac and begin to colonize the brain after embryonic day 9.5 (E9.5) 30. They exist in different interconvertible functional states. In the normal mature brain, they exist as resting ramified microglia, or “homeostatic microglia”, with highly branched processes 31,32 and are involved in a wide range of homeostatic regulation under physiological conditions 33,34, as well as in their well-known immune function 35. In response to a variety of insults, ramified microglia transform into activated phagocytic microglia that become rounded in morphology and upregulate the lysosomal protein CD68 36. Microglia also exist in the early postnatal white matter tracts as ameboid microglia with phagocytic activity before they transform into ramified microglia 37.

Nrp1 was expressed transiently on ameboid microglia in P5–8 corpus callosum and cerebellar white matter but not on ramified microglia in the cortex and became undetectable on microglia by P30. The developmental window during which Nrp1 was detected on ameboid microglia correlated with the period during which microglia-specific deletion of Nrp1 reduced OPC proliferation. Furthermore, this coincided with the period of rapid OPC proliferation and OL differentiation.

Resting ramified microglia in the mature CNS no longer expressed Nrp1. However, within three days following acute demyelination, activated CD68+ microglia/macrophages robustly upregulated Nrp1 on the cell surface. Deletion of Nrp1 from microglia significantly reduced OPC proliferation in the lesion...
during the first week after demyelination and subsequently led to reduced OL differentiation and myelination. Although the signals that dynamically upregulate Nrp1 on activated microglia in the developing and demyelinated white matter remain unknown, it is possible that phagocytosis of dead OL lineage cells by ameboid/activated microglia, a process which has been previously reported, could trigger a response that activates their Nrp1 expression.

Transcriptomic similarities between early postnatal ameboid microglia and demyelination-induced activated microglia. Recent single-cell transcriptomic studies have identified a transcriptionally unique subtype of microglia/monocytic cells in the mouse brain that are found in early postnatal white matter, which are referred to as axon tract-associated microglia (ATM) or early postnatal proliferative region-associated microglia (PAM), where oligodendrogiogenesis is actively occurring prior to myelination. Intriguingly, among microglia from different ages and from demyelinated lesions, Nrp1 transcript counts were highest in those from P4/5 brain and LPC-induced white matter lesions, whereas Nrp1 expression was lower in the homeostatic microglia in the mature CNS. Thus, the two subpopulations of phagocytic microglia not only share their transcriptome but also regulate OPC proliferation similarly by dynamically modulating Nrp1 expression on their surface.

Beneficial effects of microglia on OL/myelin production. Traditionally, microglia have been associated with inflammation and deleterious effects on OLs and myelin in demyelinating lesions of multiple sclerosis. However, beneficial effects of microglia during myelin repair have also been identified. Their dichotomous role appears to depend on their activation state, which has been previously reported. A key inducer of OPC proliferation during development and after demyelination. Developmental deletion of microglial Nrp1 did not lead to permanent myelin defects, which could be due to compensation by proliferation and migration of microglia that had escaped Nrp1 deletion. Since we only deleted Nrp1 from microglia and not from vascular cells or axons, the partial inhibition of OPC proliferation in the mg-Nrp1-cko could be due to additional effects of Nrp1 on cell types other than microglia, as well as Nrp1-independent mechanisms that also promote developmental OPC proliferation.

Nrp1 loss from activated microglia that appeared in the demyelinated lesion led to a severe decrease in OPC proliferation followed by decreased OL regeneration and protracted remyelination. With compromised proliferative response of OPCs after acute demyelination, additional OPCs may have to be recruited from surrounding areas such as the subventricular zone or the gray matter, which could have contributed to the delayed remyelination. Interestingly, Nrp1 is highly expressed by microglia/macrophages associated with malignant glioma, and deletion of Nrp1 from microglia and macrophages slows glioma growth.

Mechanism by which microglial Nrp1 promotes OPC proliferation. Nrp1 is the axonal co-receptor for class III Semaphorins and is critical for growth cone collapse induced by Sema3A. Nrp1 was highly expressed on axons in E18.5 corpus callosum but the level had significantly dropped by P5. Axonal Nrp1 became undetectable in the corpus callosum after P14 and was not re-expressed after demyelination. Thus, axonal Nrp1 is not likely to be a major modulator of OPC proliferation. A related protein Nrp2 shares 44% amino acid identity with Nrp1 and binds primarily Sema3F. Semaphorins have been implicated in OPC migration during development and after demyelination, acting via Nrp1 and 2 on OPCs. However, we did not detect Nrp1 or 2 expression on OPCs, or on other cells in the corpus callosum, which was consistent with RNA-seq data for OPCs. Neither did we detect Nrp2 on microglia, also consistent with the transcriptomic studies. Furthermore, the dramatic decrease in OPC proliferation in demyelinated lesions after Nrp1 deletion from activated microglia supports the view that microglial Nrp1 functions as a key inducer of OPC proliferation.

Different saturation levels of Nrp1 in gray and white matter differentially affect OPC proliferation. In slice cultures, blocking Nrp1 reduced PDGF AA-mediated OPC proliferation in the corpus callosum but not in the cortex. By contrast, addition of Nrp1-Fc augmented PDGF AA-mediated OPC proliferation in

![Fig. 8 Proposed mechanism of action of microglial Nrp1 on OPC proliferation](image-url)
gray but not white matter. Nrpl1 may be present in satirizing amounts in white matter, whereas in gray matter, the level of endogenous Nrpl1 may be insufficient to adequately activate PDGFRα on OPCs. This is consistent with the immunohistochemical detection of higher levels of Nrpl1 on activated microglia in white matter. This is also supported by the ability of Nrpl1-Fc to augment proliferation and PDGFRα phosphorylation on dissociated OPCs in the presence of a suboptimum concentration of PDGF AA.

Trans-activation of PDGFRα on OPCs by Nrpl1 on adjacent microglia. On mesenchymal stem cells, Nrpl1 forms a complex with PDGFRα in the presence of PDGF and increases their migration and proliferation in response to PDGFR AA.68 In this case, both Nrpl1 and PDGFRα are expressed in cis on the same cell, similar to the role of Nrpl1 in VEGF165-mediated VEGFR2 stimulation (reviewed in refs. 22,25). By contrast, our findings support a model of trans-activation of PDGFRα on OPCs by Nrpl1 on adjacent microglia (Fig. 8). Juxtacrine interaction between Nrpl1-expressing tumor cells and VEGFR2-endothelial cells can occur in vitro in the presence of VEGF165.26 In porcine aortic endothelial cells, presentation of human Nrpl1 in trans to VEGFR2 reduces internalization of VEGFR2 on the cell surface and causes delayed and sustained intracellular effects compared to cis-activation of VEGFR2 by Nrpl1.59 Our observation that Nrpl1-Fc co-clustered with PDGFRα on cultured OPCs and enhanced its phosphorylation is also consistent with the role of Nrpl1 in stabilizing PDGFRα on the OPC surface. It is also possible that Nrpl1 increases the availability of PDGF AA to PDGFRα by forming a ternary complex. On other cell types, Nrpl1 has been shown to be released by extracellular proteases60,61. However, if Nrpl1 released from microglia were to affect OPC proliferation, the diffusion radius would have to be limited to the immediate pericellular environment, as the effects were confined to the corpus callosum and did not extend to the cortex. Furthermore, the immunofluorescence signal for Nrpl1 was most prominent along the surface of microglia (Fig. 4D and Fig. S2). We previously reported a strikingly close apposition between an OPC and its adjacent microglia.62 These observations, along with the close apposition between Nrpl1+ microglia and OPCs observed here (Fig. 2), support a close contact-mediated interaction in trans. It is likely that Nrpl1 signal constitutes a part of a larger constellation of microglia-to-OPC/OL signaling that maintains the homeostasis of microglia and OPCs and how such interactions contribute to OL/myelin homeostasis. Future studies may be directed to exploring the signaling mechanism of the cross-talk between Nrpl1-expressing microglia and OPCs and how such interactions contribute to OL/myelin homeostasis.

Methods

Animals. We have complied with all relevant ethical regulations for animal testing and research. All animal procedures received ethical approval by the Institutional Animal Care and Use Committee of the University of Connecticut. Mice were housed in a facility with 12:12 light:dark cycle, 50% humidity, and maintained at 73.3 °F. For slice cultures, we used NG2cre/EG2/EZ bispecific transgenic mice, which were obtained by breeding NG2cre homozygous mice to Z/Eg homozygous or heterozygous mice.63 (Jackson Laboratory stock no. 008392, Tg(CAG-Bgeo/GFP)21Leb/J; RRID:IMSR_JAX:003920). The NG2cre mice used in this study had been heterozygous mice63 (Jackson Laboratory stock no. 003920, Tg(CAG-Bgeo/GFP)21Leb/J; RRID:IMSR_JAX:003920). The NG2cre mice used in this study had been bred with B6.129P2(Cg)-H2-Faslra/J; R26S (Jackson Laboratory stock no. 006943, B6.129(SJL)-P2R26SICre1Wgj/J) with an increasing portion of propylene oxide and embedding. Specimens were further filtration with Spurr resin was carried out with an ultracentrifuge at 41,000 rpm for 90 min. The pellets were washed three times with PBS to remove any excess embedding medium and mounted with Vectashield without DAPI (Vector, H-1000). To examine cell death, we applied ApopTag Red In Situ Apoptosis Detection Kit (Millipore, S7165) according to the manufacturer’s recommended procedure, after treating the tissues with pre-cooled ethanol acetate (2:1) for 5 min.

Transmission electron microscopy. After perfusion and post-fixation of LPC-injected mice at day 28 dpi, brains were washed in PBS. One-hundred micrometer thick coronal slices were obtained by slicing the brains with a Leica vibratome VT1000S. Slices containing the LPC-induced lesion were selected and embedded in 2.5% lysolecithin (LPC, Sigma, S4129) dissolved in PBS into the corpus callosum of 9- to 12-week-old mg-Nrp1-cont and mg-Nrp1-cko mice, using a Leica vibratome VT1000S. After perfusion and post-fixation with 100% epon resin. Tissue was embedded in 2.5% sucrose for at least 24 h, frozen in OCT compound (Tissue-Tek; Advin Scientific 14-373-65), and 20-μm sections were cut on a Leica CM350S cryostat. For free floating sections, 50-μm-thick coronal sections were cut with a Leica vibratome VT1000S and directly after fixation and direct rinsing.

The antibodies are listed in Table 1. Rabbit antibody against the extracellular domain of rat PDGFα receptor was prepared by Dr. William Stallcup as follows. For generating the receptor fragment needed for immunization, a C-terminal His6 sequence was added to a cDNA clone coding for the rat PDGFα receptor extracellular domain. This cDNA was ligated into the PCEP4 vector and transfected into 293 EBNA cells, followed by hygromycin B selection to obtain positive colonies. After establishment of confluent monolayers of the transfected cells, the secreted his-tagged receptor fragment was purified from serum-free culture supernatant by chromatography on Ni2+ agarose (Qagen). Authenticity of the purified material was confirmed by amino acid sequencing. Rabbit antiserum against this receptor fragment was affinity-purified on a column constructed by coupling the purified receptor fragment to cyanogen bromide-activated Sepharose CL-4B (Pharmacia). Immunofluorescence tests using frozen sections of postnatal day 10 mouse brain confirmed that the affinity-purified antibody labeled OPCs in wild type specimens, but yielded no labeling in Ng2 null specimens.

For immunolabeling, sections were rinsed in PBS, blocked in 5% normal goat serum (NGS) or 1% bovine serum albumin (BSA) containing 0.1% Triton X-100 in PBS for 1 h at room temperature, followed by incubation in the primary antibodies at 4°C overnight. The sections were rinsed three times in PBS and incubated in the secondary antibodies for 1 h at room temperature. After rinsing and mounting with Vectashield with DAPI (Vector, H-1200). To detect EdU, immunolabeled sections were washed three times with PBS and incubated in the Click reaction mixture containing 150 mM NaCl, 100 mM TrisHCl pH 7.15, 4 mM CuSO4.5H2O (Sigma, C6283), 4 ng/mL Alexa Fluor-647-conjugated azide (ThermoFisher, A10277), and 100 mM sodium ascorbate (Sigma, A4034) at room temperature for 30 min. The sections were then washed three times in PBS, stained with 5 μg/mL Hoechst 33342 in PBS (Invitrogen Molecular Probes, L33570), and mounted in Vectashield without DAPI (Vector, H-1000). To examine cell death, we applied ApopTag Red In Situ Apoptosis Detection Kit (Millipore, S7165) according to the manufacturer’s recommended procedure, after treating the tissues with pre-cooled ethanol acetate (2:1) for 5 min.

Demeyelinating lesion. To induce demyelination, mice were anesthetized using isoflurane and their heads were placed in a stereotaxic apparatus. We injected 2 μl of 2.5% lysophosphatidyl LPC (LPC, Sigma, ALX14129) dissolved in PBS into the corpus callosum of 9- to 12-week-old mg-Nrp1-cont and mg-Nrp1-cko mice, using a stereotaxic coordinates 0.3 mm anterior from the bregma, 1 mm lateral, and 1.9 mm from the surface of the skull.66 The animals were perfused at 3, 7, 14, and 28 days post lesioning (dpi). To examine OPC proliferation, EdU was injected twice, 2 h apart, before sacrifice at 3 or 7 dpi.
transmission electron microscope operated at an accelerating voltage of 80 kV and equipped with an AMT 2k (4 megapixel) XR40 CCD camera.

Slice cultures. Slice cultures from the forebrain and cerebellum were prepared from P8 NG2Cre;Z/EG mice or from P8 mg-Nrp1-cont or cko mice after 4-OHT injection at P2-3 in vivo. Forebrains were chopped into 300-µm thick slices and transferred to ice-cold dissection medium consisting of 124 mM NaCl, 2.0 mM KCl, 1.25 mM KH2PO4, 4.004 mM MgSO4 (anhydrous), 2.0 mM CaCl2⋅H2O, 26 mM NaHCO3, 10 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffer, 2 mM ascorbic acid and 0.075 mM adenosine dissolved in water. Individual slices were separated and placed on Millicell filters. After cooling on ice, brain sections were cut using a McIlwain tissue chopper into 400-µm-thick slices with the rostral cortex facing up. Slices were transferred to ice-cold dissection medium consisting of 124 mM NaCl, 2.004 mM KCl, 1.25 mM KH2PO4, 4.004 mM MgSO4 (anhydrous), 2.0 mM CaCl2 ⋅H2O, 26 mM NaHCO3, 10 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffer, 2 mM ascorbic acid and 0.075 mM adenosine dissolved in water. Individual slices were separated and placed on Millicell filters. After cooling on ice, brain sections were cut using a McIlwain tissue chopper into 400-µm-thick slices with the rostral cortex facing up. The tissue was then taken up in 2 ml DMEM-Sato medium containing 2% FBS, 10% serum, 1% NEAA, 2 mM L-glutamine, 200 U/mL penicillin, 200 µg/mL streptomycin, and 20 ng/mL P450. The tissue was then incubated at 37 °C for 30 min, after which the cells were promptly chilled on ice for 5 min. Following 6-8 times wash with D-PBS, the adhered OPCs were trypsinized for 6 min and trypsin was inactivated using 30% fetal bovine serum (FBS). Following centrifugation, the OPCs were resuspended in DMEM-Sato medium containing Dulbecco’s Modified Eagle Medium (Invitrogen), 2 mM L-glutamine, SATO supplements (1 µg/mL transferrin, 1 µg/mL BSA, 0.16 µg/mL putrescine, 0.6 ng/mL progesterone, 0.2 ng/mL sodium selenite, penicillin/streptomycin, 1 µg/mL pyruvate, 5 µg/mL insulin, 5 µg/mL N-acetyl-l-cysteine (Sigma-Aldrich, A8806), D-PBS (Invitrogen), Trypsin inhibitor (Worthington Biochemical, LS003126). The brain tissues were dissociated by gently pipetting in low-oxygenated solution. Following centrifugation, the cell pellet was suspended in panel buffer containing of 0.2% BSA and 2 mg/mL insulin in DPH and immunopanned in 10-cm petri dishes precoated with CD140a rat anti-mouse PDGFRα antibodies for 45 min. Following 6-8 times wash with D-PBS, the adhered OPCs were trypsinized for 6–7 min, and trypsin was inactivated using 30% fetal bovine serum (FBS). Following centrifugation, the OPCs were resuspended in DMEM-Sato medium containing Dulbecco’s Modified Eagle Medium (Invitrogen), 2 mM L-glutamine, SATO supplements (1 µg/mL transferrin, 1 µg/mL BSA, 0.16 µg/mL putrescine, 0.6 ng/mL progesterone, 0.2 ng/mL sodium selenite, penicillin/streptomycin, 1 µg/mL pyruvate, 5 µg/mL insulin, 5 µg/mL N-acetyl-l-cysteine (Sigma-Aldrich, A8806), 1X Tracer Elements B (Cellgro-Medical, Fisher Scientific MT99175Cl), 10 ng/mL d-Biotin (Sigma-Aldrich, B4639) and 1X B-27 (Thermo Fisher, 17504044). Purified OPCs were plated on glass coverslips coated with poly-l-lysine (Sigma, P7405, 10 µg/mL) at a density of 40,000 cells/well in 24-well plates for EdU incorporation assays or on 35-mm tissue culture dishes coated with poly-l-lysine (Sigma, P7405, 10 µg/mL) at a density of 10 × 10^4 cells/well for immunofluorescence labeling and EdU detection.

Dissociated OPC cultures. OPCs from the cerebral cortex of P4-5 wild type CD1 mice were immunoaffiliated with PDGFRα using CD140a rat anti-mouse PDGFRα antibody. The brains were scooped out from the opened skull and diced in an ice-cold D-PBS without Ca2+ and Mg2+ (Invitrogen) in the dish. The minced tissue was incubated in an oxygenated perfusion solution containing 20 units of papain (Worthington Biochemical, LS003126), 2 mg of l-cysteine (Sigma-Aldrich, A8806) and DMEase I (Worthington Biochemical, L00207) on a 37 °C heating block for 30–45 min. Following tissue digestion, papain was inactivated using a low-oxygen solution containing BSA (Sigma-Aldrich, A8806), D-PBS (Invitrogen), Trypsin inhibitor (Worthington Biochemical, LS003126). The brain tissues were dissociated by gently pipetting in low-oxygen solution. Following centrifugation, the cell pellet was suspended in panel buffer containing of 0.2% BSA and 2 mg/mL insulin in DPH and immunopanned in 10-cm petri dishes precoated with CD140a rat anti-mouse PDGFRα antibodies for 45 min. Following 6-8 times wash with D-PBS, the adhered OPCs were trypsinized for 6–7 min, and trypsin was inactivated using 30% fetal bovine serum (FBS). Following centrifugation, the OPCs were resuspended in DMEM-Sato medium containing Dulbecco’s Modified Eagle Medium (Invitrogen), 2 mM L-glutamine, SATO supplements (1 µg/mL transferrin, 1 µg/mL BSA, 0.16 µg/mL putrescine, 0.6 ng/mL progesterone, 0.2 ng/mL sodium selenite, penicillin/streptomycin, 1 µg/mL pyruvate, 5 µg/mL insulin, 5 µg/mL N-acetyl-l-cysteine (Sigma-Aldrich, A8806), 1X Tracer Elements B (Cellgro-Medical, Fisher Scientific MT99175Cl), 10 µg/mL d-Biotin (Sigma-Aldrich, B4639) and 1X B-27 (Thermo Fisher, 17504044). Purified OPCs were plated on glass coverslips coated with poly-l-lysine (Sigma, P7405, 10 µg/mL) at a density of 40,000 cells/well in 24-well plates for EdU incorporation assays or on 35-mm tissue culture dishes coated with poly-l-lysine (Sigma, P7405, 30 µg/mL) at a density of 10 × 10^4 cells/well for immunofluorescence labeling. For EdU detection, the cells on coverslips were initially incubated in 1X EdU labeling solution for 5 h in the dark. To visualize EdU incorporation, cell nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI). Following fixation, the cells were incubated with a primary antibody (R&D Systems, AF1062) during the last 48 h of incubation (on day 7). Nrp1-Fc fusion protein containing the extracellular domain of rat Nrp1 amino acid 22-854 minus amino acid 24-828 (R&D Systems, AF1062) during the last 5 h of incubation. At the end of the incubation, slices were fixed with 4% paraformaldehyde and processed for immunofluorescence labeling and EdU detection.
Received: 16 January 2020; Accepted: 8 March 2021; Published online: 15 April 2021

References

1. Dawson, M. R., Polito, A., Levine, J. M. & Reynolds, R. NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Mol. Cell Neurosci. 24, 476–488 (2003).

2. Nishiyama, A., Komitova, M., Suzuki, R. & Zhu, X. Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nat. Rev. Neurosci. 10, 9–22 (2009).

3. Nishiyama, A., Boshons, L., Goncalves, C. M., Wegryn, J. & Patel, K. D. Lineage, fate, and fate potential of NG2-glia. Brain Res. 1638, 116–128 (2016).

4. Richardson, W. D., Pringle, N., Musol, M. J., Westermark, B. & Dubois-Dalcq, M. A role for platelet-derived growth factor in normal glio genesis in the central nervous system. Cell 53, 309–319 (1988).

5. Calver, A. R. et al. Oligodendrocyte population dynamics and the role of PDGF in vivo. Neuron 20, 689–692 (1998).

6. Frutiger, M., Calver, A. R. & Richardson, W. D. Platelet-derived growth factor is constitutively secreted from neuronal cells but not from axons. Curr. Biol. 10, 1283–1286 (2000).

7. Wu, Q. et al. Elevated levels of the chemokine GRO-1 correlate with elevated oligodendrocyte progenitor proliferation in the jimpy mutant. J. Neurosci. 20, 2609–2617 (2000).

8. Bu, J., Banki, A., Wu, Q. & Nishiyama, A. Increased NG2(+) glial cell proliferation and oligodendrocyte generation in the hypomyelinating mutant shiverer. Glia 48, 51–63 (2004).

9. Gersnt, J. M. & Goldman, J. E. Endogenous progenitors remyelinate demyelinated axons in the adult CNS. Neuron 19, 197–203 (1997).

10. Keirstead, H. S., Levine, J. M. & Blakemore, W. F. Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. Glia 22, 161–170 (1998).

11. Di Bello, C. I., Dawson, M. R., Levine, J. M. & Reynolds, R. Generation of oligodendroglial progenitors in acute inflammatory demyelinating lesions of the rat brain stem is associated with remyelination rather than inflammation. J. Neurocytol. 28, 365–389 (1999).

12. Watanebe, M., Toyama, Y. & Nishiyama, A. Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. J. Neurosci. Res. 69, 826–836 (2002).

13. Hill, R. A. & Nishiyama, A. NG2 cells (polydendrocytes): listeners to the neural network with diverse properties. Glia 62, 1195–1210 (2014).

14. Boshans, L. L., Sheratif, A. & Nishiyama, A. The effects of developmental and current niches on oligodendrocyte precursor dynamics and fate. Neurosci. Lett. 715, 134593 (2020).

15. Hill, R. A., Patel, K. D., Medved, J., Reiss, A. M. & Nishiyama, A. NG2 cells in white matter but not gray matter proliferate in response to PDGF. J. Neurosci. 33, 14558–14566 (2013).

16. Vignano, F., Mobius, W., Gotz, M. & Dimou, L. Transplantation reveals regional differences in oligodendrocyte differentiation in the adult brain. Nat. Neurosci. 16, 1370–1372 (2013).

17. Andrae, J., Gallini, R. & Besholtz, C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev. 22, 1276–1312 (2008).

18. Ball, S. G., Bayley, C., Shuttlesworth, C. A. & Kirby, C. M. Neurophin-1 regulates platelet-derived growth factor receptor signalling in mesenchymal stem cells. Biochem. J. 427, 29–40 (2010).

19. Takagi, S. et al. The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron 7, 295–307 (1991).

20. Kawakami, A., Katsukawa, T., Takagi, S. & Fujisawa, H. Developmentally regulated expression of a cell surface protein, neurophin, in the mouse nervous system. J. Neurobiol. 29, 1–17 (1996).

21. He, Z. & Tessier-Lavigne, M. Neurophin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90, 739–751 (1997).

22. Zachary, I. C. How neurophin-1 regulates receptor tyrosine kinase signalling: the knowns and known unknowns. Biochem. Soc. Trans. 39, 1583–1591 (2011).

23. Austyn, J. M. & Gordon, S. Fl50a, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. 11, 805–815 (1981).

24. Holness, C. L. & Simmons, D. L. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood 81, 1607–1613 (1993).

25. Hughes, M. M., Field, R. H., Perry, V. H., Murray, C. L. & Cunningham, C. Microglia in the degenerating brain are capable of phagocytosis of cells and of apoptotic cells, but do not efficiently remove PrPSc, even upon E6 expression. Glia 58, 2017–2030 (2010).
26. Gu, C. et al. Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. Dev. Cell 5, 45–57 (2003).

27. Parkhurst, C. N. et al. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. Cell 155, 1596–1609 (2013).

28. Zhang, Y. et al. An RNA-sequencing transcriptome and splicing database of gli, neurons, and vascular cells of the cerebral cortex. J. Neurosci. 34, 11392–11407 (2014).

29. Shigemoto-Mogami, Y., Hoshikawa, K., Goldman, J. E., Sekino, Y. & Sato, K. Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone. J. Neurosci. 34, 2231–2243 (2014).

30. Gámez, L. E. et al. The role of metabotropic glutamate receptors in the nervous system. Trends Neurosci. 11, 273–277 (1988).

31. Perry, V. H. & Gordon, S. Macrophages and microglia in the nervous system. Physiol. Rev. 91, 461–553 (2011).

32. Kettenmann, H., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. J. Neurosci. 34, 132 (2014).

33. Chi, J., Hayashi, T. & Kominami, E. Role of microglia in neurodegenerative and inflammatory diseases. Cell. Mol. Life Sci. 73, 4305–4318 (2016).

34. Butovsky, O. & Weiner, H. L. Microglial signatures and their role in health and disease. Nat. Rev. Neurosci. 19, 622–635 (2018).

35. Owens, T. Immune functions of microglia. in Neuroglia (ed. Ransom, H.K.A.B.R.) 638–648 (Oxford University Press, New York, 2013).

36. Streit, W. J. in Immunity, Inflammation, and Disease, 2nd edn (ed. Ransom, H.K.A.B.R.) 86–97 (Oxford University Press, New York, 2013).

37. Ling, E. A. Some aspects of amoeboid microglia in the corpus callosum and neighbouring regions of neonatal rats. J. Anat. 121, 29–45 (1976).

38. Ellison, J. A. & de Vellis, J. Amoeboid microglia expressing CD11b, ganglioside GM1 are concentrated in regions of oligodendrogenesis during development of the corpus callosum. J. Comp. Neurol. 304, 123–132 (1990).

39. Hammond, T. R. et al. Single-cell RNA sequencing of microglia throughout the mouse lifespan and in the injured brain reveals complex cell-state changes. Immunity 50, 253–271 (2019).

40. Li, Q. et al. Developmental heterogeneity of microglia and brain myeloid cells revealed by deep single-cell RNA sequencing. Neuron 101, 267–223 (2019).

41. Lassmann, H. Multiple sclerosis lesions from molecular neuropathology. Exp. Neurol. 262, 2–7 (2014).

42. Miron, V. E. et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat. Neurosci. 16, 1211–1218 (2013).

43. Nicholas, R. S., Wing, M. G. & Compton, A. Nonactivated microglia promote oligodendrocyte precursor survival and maturation through the transcription factor NF-kappa B. Eur. J. Neurosci. 13, 959–967 (2001).

44. Butovsky, O. et al. Induction and blockade of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. J. Clin. Invest. 116, 905–915 (2006).

45. Dodson, A. A novel microglial subset plays a key role in myelogenesis in developing brain. EMBO J. 36, 3202–3208 (2017).

46. Hagemeyer, N. et al. Microglia contribute to normal myelogenesis and to oligodendrocyte progenitor maintenance during adulthood. Acta Neuropathol. 134, 441–458 (2017).

47. Erblin, B., Zhu, L., Egen, A. M., Dobrenis, K. & Pollard, J. W. Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits. PLoS ONE 6, e26317 (2011).

48. Caponegro, M. D., Moffitt, R. A. & Tsuchiya, S. E. Expression of neuropilin-1 is linked to glioma associated microglia and macrophages and correlates with unfavorable prognosis in high grade gliomas. Oncotarget 9, 35655–35665 (2018).

49. Miyachi, J. T. et al. Deletion of neuropilin 1 from microglia or bone marrow-derived macrophages slows glia progression. Cancer Res. 78, 685–694 (2018).

50. Koldobkin, A. L. et al. Neuropilin is a semaphorin III receptor. Cell 90, 753–762 (1997).

51. Luo, Y., Raible, D. & Raper, J. A. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75, 217–227 (1993).

52. Sugimoto, Y. et al. Guidance of glial precursor cell migration by secreted cues in the developing optic nerve. Development 128, 3321–3330 (2001).

53. Spassky, N. et al. Directional guidance of oligodendroglial migration by class 3 semaphorins and netrin-1. J. Neurosci. 22, 5992–6004 (2002).

54. Williams, A. et al. Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis? Brain 130, 2554–2565 (2007).

55. Piaton, G. et al. Class 3 semaphorins influence oligodendrocyte precursor recruitment and remyelination in adult central nervous system. Brain 134, 1156–1167 (2011).
