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## Regional diversity in oligodendrocyte progenitor cells

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# Chapter 3

**Grey matter oligodendrocyte lineage cells are more dependent on signaling via the primary cilium than white matter oligodendrocyte lineage cells**



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## Abstract

Remyelination upon demyelination is essential for functional recovery but often fails in later stages of the chronic inflammatory, demyelinating and neurodegenerative disease multiple sclerosis. Efficient remyelination requires proliferation and differentiation of oligodendrocyte progenitor cells (OPCs). Remyelination is more robust in grey matter lesions than white matter lesions, a finding that may be attributed to a different micro-environment and/or OPC diversity. A key organelle transducing micro-environmental signals into altered cell behavior is the primary cilium. This cell signaling hub is displayed on OPCs and is involved in the initiation of sonic hedgehog-mediated modulation of PDGF-induced proliferation. Here, we investigated whether primary cilium formation on grey and white matter OPCs differs in the developing brain and upon demyelination, and in their function in OPC proliferation and differentiation *in vitro*. More oligodendrocyte lineage cells in the developing cortex display a primary cilium than in the corpus callosum, and the percentage of primary cilia-displaying oligodendrocyte lineage cells tend to increase upon demyelination in the cortex. *In vitro* findings indicate that this organelle is carried earlier on differentiating grey matter OPCs, while being most prominently displayed on grey and white matter-derived immature oligodendrocytes. Preventing primary cilia formation by a knockdown of the intraflagellar transport protein IFT88 revealed that primary cilia are involved in mitogen-induced grey matter, but not white matter, OPC proliferation and Wnt3a-mediated differentiation of grey matter immature oligodendrocytes. Our findings indicate that signaling via the primary cilium may be indispensable for regulating proliferation and differentiation of grey matter oligodendrocyte lineage cells, while being dispensable in white matter oligodendrocyte lineage cells. This difference in the processing of environmental signals may contribute to regional differences in remyelination efficiency.

## Introduction

Remyelination is a natural repair process upon demyelination in which new myelin sheaths are formed by newly-differentiated oligodendrocyte progenitor cells (OPCs)<sup>3</sup> or pre-existing mature oligodendrocytes (OLGs)<sup>22</sup>. Regeneration of myelin is essential for restoring saltatory conduction<sup>393</sup> and preventing neurodegeneration<sup>394</sup>. In the chronic disease multiple sclerosis (MS), persistent demyelinated lesions develop in both the grey matter (GM) and white matter (WM) of the central nervous system (CNS). Remyelination in GM MS lesions is more robust than remyelination in WM MS lesions<sup>26,27,353</sup>, a finding that is also observed in an experimental model for de- and remyelination<sup>24,25</sup>. The microenvironment in demyelinated GM lesions may be more permissive of remyelination due to a higher OPC density<sup>25-27</sup>, a different extracellular matrix composition<sup>26,270</sup> and different spatial and temporal expression of growth factors<sup>244</sup> and inflammatory signals<sup>32,255,347,354,355</sup> compared to demyelinated WM lesions. Moreover, recently we and others have described intrinsic differences in OPCs from the GM (gmOPCs) and WM (wmOPCs). GmOPCs are more immature than wmOPCs<sup>38,44,144</sup>, are morphologically less complex<sup>41,138,144</sup>, differentiate less efficiently into mature OLGs<sup>38,39,44,144</sup>, and have higher numbers of AMPA/kainate receptors and lower numbers of NMDA receptors compared to wmOPCs<sup>37</sup>. In addition, gmOPCs express lower levels of OLG maturation genes and are less susceptible to pro-inflammatory cytokine-mediated inhibition of OPC differentiation<sup>144</sup>. Given that OPCs revert to a more immature state before successful remyelination<sup>57</sup>, gmOPCs might be better equipped for remyelination.

Diversity in response to molecular signals, and the susceptibility thereto in gmOPCs and wmOPCs may contribute to regional differences in remyelination efficiency. Various extracellular signals are transduced to intracellular signaling pathways via cilia. Cilia are cell organelles on eukaryotic cells that come in two forms: motile and non-motile cilia. Motile cilia can be present in large numbers on cells and by moving in a coordinated wave-like fashion reallocate debris and mucus in the lungs<sup>395</sup> and push cerebrospinal fluid in the CNS<sup>396</sup>. In contrast, non-motile cilia, also called primary cilia, usually manifest as a single cilium on cells serving as a signaling hub for soluble extracellular signals including sonic hedgehog (Shh)<sup>397</sup>, Wingless and integration site (Wnt)<sup>398-401</sup>, although controversial, see <sup>402</sup> and references therein), platelet-derived

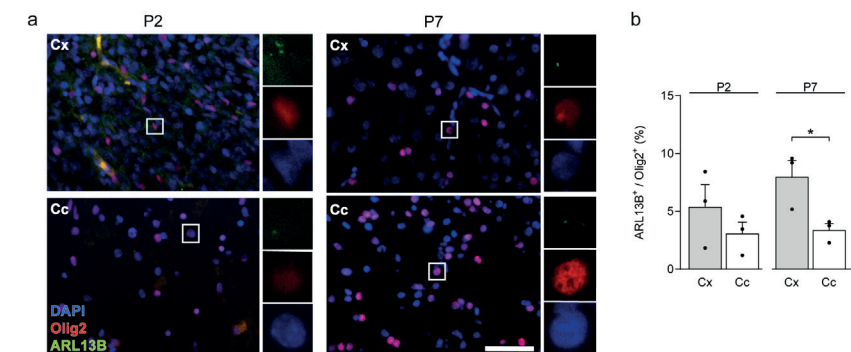
growth factor (PDGF)<sup>403</sup>, fibroblast growth factor (FGF)<sup>404</sup>, Notch<sup>405</sup>, and mTor<sup>406</sup>, and have also been linked to signaling via mechanosensation and extracellular matrix molecules. These signaling events have been implicated in processes that are essential for successful remyelination, including OPC proliferation, migration and differentiation (reviewed in <sup>49,407,408</sup>). This indicates that primary cilia may be important in regulating OPC behavior. A previous study by Falcón-Urrutia and co-workers showed that a primary cilium is displayed on primary grey matter OPCs and immature OLGs that is functionally involved Shh-mediated modulation of PDGF-induced proliferation<sup>59</sup>. The abundance and function of primary cilia on developing primary wmOPCs was not investigated. However, primary cilia are detected in the developing corpus callosum and cortex, and analysis of all cells revealed that more cells carry the organelle in the neonatal cortex than in the adult cortex, while numbers in the corpus callosum were not compared<sup>409</sup>.

Here we set out to investigate whether there is a difference in the percentage of primary cilia-displaying oligodendrocyte lineage cells of GM and WM *in vitro*, *in vivo* and upon demyelination. In addition, a potential role of primary cilia in PDGF-AA- and FGF-2-mediated OPC proliferation and Wnt3a-mediated modulation of OPC differentiation was examined, and whether this may differ between gmOPCs and wmOPCs. Our findings revealed that the percentage of oligodendrocyte lineage cells that display a primary cilium was higher in the cortex than the corpus callosum in developing CNS, upon CNS demyelination, and displayed earlier in differentiating primary gmOPCs than wmOPCs. In addition, the primary cilium was required for transducing extracellular signals for proliferation and differentiation in primary gmOPCs and immature gmOLGs, but not wmOPCs and immature wmOLGs. This distinct organization of signaling responses may contribute to the regional differences in remyelination efficiency.

## Results

### More oligodendrocyte lineage cells display a primary cilium in the cortex than in the corpus callosum at postnatal day 7

In rodents, the cortex is populated around birth by the third and final wave of OPCs and these OPCs migrate in the first postnatal week locally to the corpus callosum, making up most of the oligodendrocyte lineage in the cortex and corpus callosum in the adult<sup>60,160</sup>. To examine whether primary cilia are differentially displayed on oligodendrocyte lineage cells in the cortex and the corpus callosum, the colocalization of primary cilia with the oligodendrocyte lineage marker Olig2 was quantified in both regions around birth, i.e., at postnatal day 2 (P2), and at P7, i.e., just before the onset of developmental myelination and when the distribution of the OPC population approaches a steady-state<sup>60</sup>. Primary cilia are observed as single 1-3  $\mu\text{m}$  long rods on cells when immunostained for ADP-ribosylation factor-like protein 13B (ARL13B);

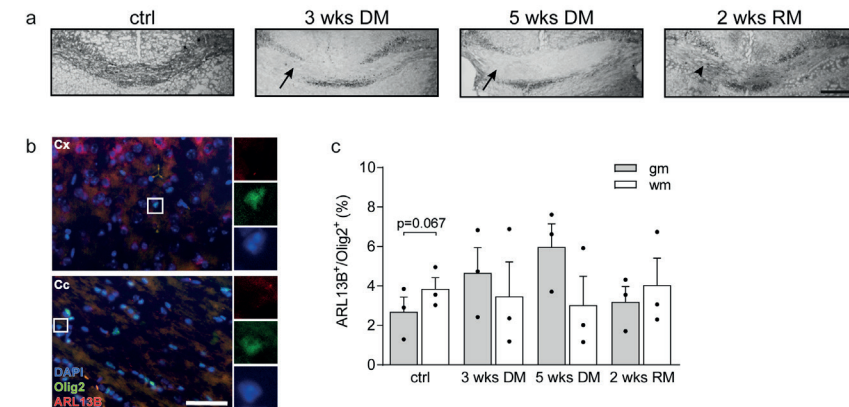


**Figure 1.** More oligodendrocyte lineage cells in the cortex display a primary cilium than oligodendrocyte lineage cells in the corpus callosum at postnatal day 7. **(a,b)** Primary cilia (ARL13B, green) localization with oligodendrocyte lineage cells (Olig2, red) was quantified in sections of the cortex (cx) and corpus callosum (cc) of postnatal day 2 (P2) and P7 rats. Representative images are shown in **a**. Quantitative analysis of the percentage of Olig2-positive cells that displayed primary cilia (ARL13B) in cortex (grey bars) and corpus callosum (white bars) in **b**. Note that while the percentage of primary cilia-displaying oligodendrocyte lineage cells remains stable from P2 to P7 in corpus callosum, the percentage is increased in cortex at P7 compared to P2. Bars represent absolute mean of the percentage of Olig2-positive cells with an ARL13B-positive cilium in cortex (grey bars) and corpus callosum (white bars). Error bars show the standard error of the mean. Statistical analyses are performed using a paired two-sided t-test to test for differences between cortex and corpus callosum at the indicated postnatal day **(b)** (\* $p < 0.05$ ). Scale bar is 50  $\mu\text{m}$  **(a)**.

a ciliary G protein of the Ras superfamily. At P2, about 3-5% of the Olig2-positive cells in cortex and corpus callosum harbor a primary cilium (Fig. 1a,b). At P7, the percentage of Olig2-positive cells that display a primary cilium was increased in the cortex, while the percentage remained similar in the corpus callosum. In fact, the percentage of Olig2-positive cells in the P7 cortex that present a primary cilium was significantly higher than in the P7 corpus callosum (Fig. 1a,b  $p=0.038$ ). To investigate whether primary cilia are similarly distinctly displayed on oligodendrocyte lineage cells in the cortex and corpus callosum upon demyelination, primary cilia formation on Olig2-positive cells during demyelination and remyelination was examined next.

### Primary cilia-displaying oligodendrocyte lineage cells slightly increase in the cortex, but not in the corpus callosum upon demyelination

The dietary cuprizone model is a well-established experimental toxin-induced model to study de- and remyelination in the CNS<sup>410</sup>. The copper chelator cuprizone is selectively toxic to OLGs and induces global demyelination in the brain, including cortex and corpus callosum, which is maximal after 5 weeks cuprizone feeding<sup>24,25</sup>. Return to a normal diet results in robust remyelination by endogenous OPCs within 2 weeks<sup>24,25</sup>. To investigate whether primary cilium dynamics is altered in oligodendrocyte lineage cells during upon cuprizone-induced demyelination, the colocalization of primary cilia with Olig2-expressing cells was quantified in the corpus callosum and cortex at 3 and 5 weeks demyelination and at 2 weeks remyelination. The myelin stain Sudan black verified demyelination at 3 (3 wks DM) and 5 (5 wks DM) weeks of cuprizone feeding, and remyelination after 5 weeks cuprizone feeding and 2 weeks (2 wks RM) normal feeding (Fig. 2a). In control young adult mice brain, approximately 2% of the Olig2-expressing cells in the cortex display a primary cilium compared to approximately 4% in the corpus callosum (Fig. 2b,c  $p=0.067$ ). Upon demyelination, colocalization of primary cilia with Olig2-expressing cells substantially, but not significantly, increased almost 3-fold at 5 weeks cuprizone feeding in the cortex, while the percentage Olig2-expressing cells that harbor primary cilia remained similar upon demyelination (Fig. 2b,c). At 2 weeks remyelination, the percentage of primary cilia-harboring oligodendrocyte lineage cells in the cortex returned to levels as observed in the cortex of control mice (Fig. 2b,c). Hence, upon demyelination, the percentage of primary cilia-displaying oligodendrocyte lineage



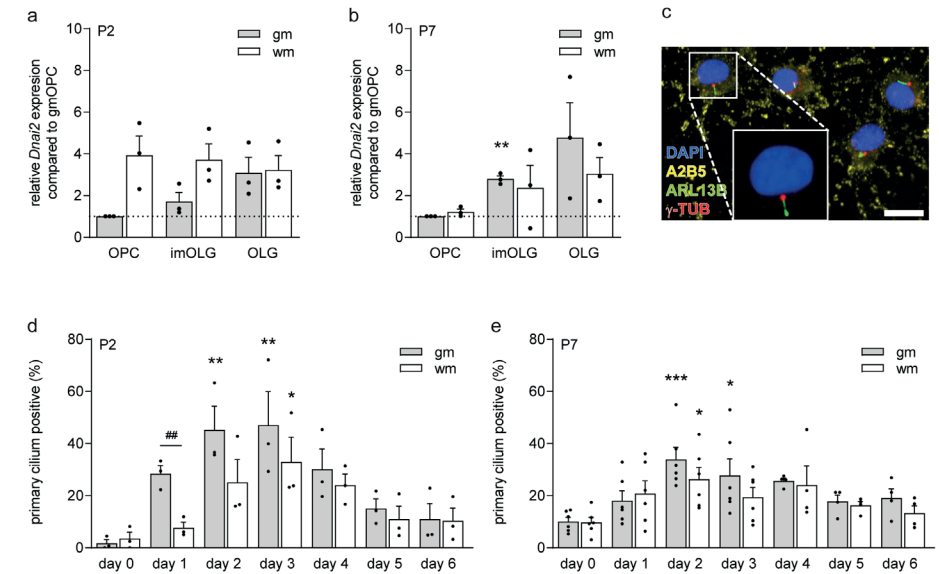
**Figure 2.** Primary cilia-displaying oligodendrocyte lineage cells slightly increase in the cortex, but not in the corpus callosum upon demyelination. Mice were fed with normal chow (ctrl) or fed with chow supplemented with cuprizone for 3 (3 wks DM) or 5 (5 wks DM) weeks to induce demyelination, after which remyelination was induced by switching to normal chow for 2 weeks (2 wks RM). (a) Histological staining with the myelin stain Sudan black. Note the prominent demyelination at 3 and 5 weeks of cuprizone feeding (arrows). Representative images are shown. (b,c) Primary cilia (ARL13B, red) localization with oligodendrocyte lineage cells (Olig2<sup>+</sup>, green) was quantified in sections of the cortex (cx, grey bars) and corpus callosum (cc, white bars). Representative images of control animals are shown in b and quantification in c. Note that while the percentage of primary cilia-carrying oligodendrocyte lineage cells remain largely unaltered in the corpus callosum, in the cortex the percentage is slightly increased upon demyelination. Bars represent absolute mean of the percentage of Olig2<sup>+</sup> cells with an ARL13B<sup>+</sup> cilium in cortex (grey bars) and corpus callosum (white bars). Error bars show the standard error of the mean. Statistical analyses are performed using an one-way ANOVA with Dunnett post-test for differences in cx or cc over time, or a paired two-sided t-test to test for differences between cortex and corpus callosum at the indicated postnatal day (c) (not significant). Scale bars are 200  $\mu$ m (a) and 50  $\mu$ m (b).

cells tended to transiently increase in the cortex, but not in corpus callosum. To examine the function of primary cilia on OPCs and differentiating oligodendrocyte lineage cells, the presence of primary cilia on primary differentiating neonatal gmOPCs and wmOPCs was investigated next.

### More P2-derived immature gmOLGs display a primary cilium than differentiating P2-derived immature wmOLGs upon *in vitro* OPC differentiation

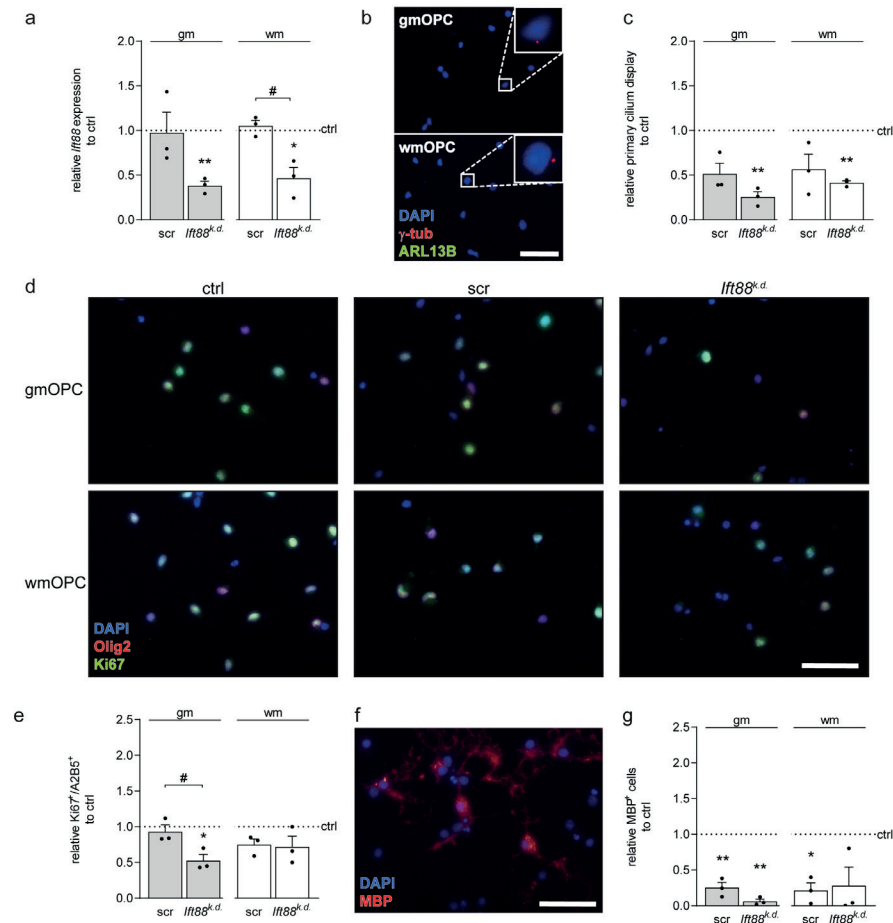
To assess formation of primary cilia on differentiating oligodendrocyte lineage cells, primary neonatal cortical OPCs (referred to as gmOPCs) and non-cortical OPCs (mainly WM, referred to as wmOPCs) were obtained of newborn rats at P2 and P7. As

shown in figure 3a, a subset of primary A2B5-positive OPCs display a single primary cilium. Upon initiating OPC differentiation distinct developmental stages can be distinguished, and include morphological differentiation from a bipolar OPC to a multiple and branched process containing immature OLGs 3 days after initiating differentiation and finally into myelin membrane-bearing mature OLGs 6 days after initiating differentiation<sup>88,411</sup>. Dynein axonemal intermediate chain 2 (DNAI2) is a protein involved in the function of axoneme of primary cilia<sup>412</sup> of which transcripts are highly expressed in gm-derived immature OLGs<sup>86</sup>. To investigate whether mRNA expression levels of the gene encoding DNAI2 (*Dnai2*) differ upon gmOPC and wmOPC differentiation, qPCR analysis was performed. *Dnai2* transcript levels were reproducibly increased upon differentiation of P2- and P7-derived gmOPCs, but not of their wmOPC counterparts, compared to the transcript levels at the onset of differentiation (Fig. 3a,b, P7 gm imOLG  $p=0.007$ ). This indicates that primary cilia may play a more prominent role in regulating gmOPC behavior. To assess whether the percentage of primary cilia-harboring cells is altered during *in vitro* differentiation, the percentage of cells that display a primary cilium were quantified daily after initiating OPC differentiation using anti-ARL13B and anti-gamma-tubulin ( $\gamma$ -tub) (Fig. 3c). Of note, anti- $\gamma$ -tub stains centrioles from which primary cilia extend<sup>413</sup>. Upon P7-derived OPC differentiation, the percentage of cells that harbor a primary cilium transiently increased 2-3 fold with a peak abundance on immature OLGs, i.e., 2 and 3 days after initiating differentiation (Fig. 3d). While the percentage of primary cilia-harboring gm-derived immature OLGs significantly increased compared to gmOPCs (Fig. 3d, day 2  $p=0.001$ , day 3  $p=0.015$ ), no differences were observed in the percentage of cells that displayed a primary cilium on differentiating P7-derived GM oligodendrocyte lineage cells compared to WM oligodendrocyte lineage cells (Fig. 3e). A more prominent transient increase in primary cilia-displaying cells on differentiating P2-derived gmOPCs and wmOPCs was observed (Fig. 3d). More specifically, the percentage of primary cilia-displaying cells was significantly increased two days after gmOPC differentiation (Fig. 3e,  $p=0.004$ ) and three days after initiating both gmOPC and wmOPC differentiation (Fig. 3e, gmOPC  $p=0.003$ , wmOPC  $p=0.016$ ) compared to day 0. In addition, the increase in percentage of primary cilium-harboring cells was earlier observed upon P2-derived gmOPC than wmOPC differentiation (Fig. 3e,  $p=0.007$ ). Notably, and in contrast to previous findings that found 80% of OPCs display a primary cilium at the onset of differentiation<sup>59</sup>, in our synchronized cultures,



**Figure 3.** More P2-derived immature gmOLGs display a single primary cilium than differentiating P2-derived immature wmOLGs upon *in vitro* OPC differentiation. Oligodendrocyte progenitor cells (OPCs) isolated from postnatal day 2 (P2) or P7 rat brain cortex (gmOPCs) and non-cortex (wmOPCs) were allowed to differentiate into mature oligodendrocytes for 6 days. Primary cilia display on OPCs was quantified daily. (a,b) Relative expression of *Dnai2* in OPCs, immature oligodendrocytes (imOLG) and mature oligodendrocytes (OLG) from GM (grey bars) and WM (white bars). Expression is made relative to the expression in gmOPCs, which is set to 1 (dashed line). (c) Representative image of primary cilia that are observed as rod-like structures (ARL13B, green) with a centrosomic anchor (gamma-tubulin ( $\gamma$ -tub), red) on A2B5-positive oligodendrocyte progenitor cells (OPCs, yellow). (d) Quantitative analysis of primary cilium display on DAPI-stained P7-derived gmOPCs (grey bars) and wmOPCs (white bars) that were allowed to differentiate for 1 to 6 days *in vitro*. (e) Quantitative analysis of primary cilium display on P2-derived gmOPCs (grey bars) and wmOPCs (white bars) that were allowed to differentiate for 1 to 6 days *in vitro*. Note that upon OPC differentiation the percentage of primary cilia-displaying cells is transiently increased at the immature oligodendrocyte stage, i.e., 2-3 days after initiating differentiation, and that differentiating P2-derived gmOPCs harbor primary cilia earlier than differentiating P2-derived wmOPCs upon differentiation. Bars represent relative expression of *Dnai2* to gmOPCs, which is set to 1 (a,b), and absolute mean of the percentage of cells with primary cilium (c,d), in differentiating gmOPCs (grey bars) and wmOPCs (white bars). Error bars show the standard error of the mean. Statistical analyses are performed using a one-sample t-test to compare with gmOPCs (a), or with an ANOVA with a Tukey post-test to test for differences in gmOPCs or wmOPCs in time (a,c), indicated with \*, or with a paired t-test to compare between gmOPCs and wmOPCs, indicated with # (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ ). Scale bar is 50  $\mu$ m (c).

10% of the P7-derived OPCs contained a primary cilium at day 0, while only 3% of the P2-derived OPCs display a primary cilium at the onset of differentiation (Fig. 3d,e). Hence, upon OPC differentiation the percentage of primary cilia-displaying



**Figure 4.** Primary cilia are involved in PDGF-AA- and FGF-2-mediated gmOPC proliferation, as well as gmOPC differentiation. *Ift88*, essential for primary cilium formation, was knocked down by short-hairpin RNA interference (*Ift88<sup>kd</sup>*) in mixed glial cultures from cortex (grey matter; GM) and non-cortex (white matter; WM) obtained from newborn rats (postnatal day 2). Oligodendrocyte progenitor cells (OPCs) were shaken off and cultured for 48 hours in the presence of mitogens PDGF-AA and FGF-2 or allowed to differentiate for 3 or 6 days. Non-transduced cells (ctrl) and cells transduced with scrambled (scr) shRNA served as controls. (a) *Ift88* mRNA expression levels were quantified by qPCR after 48 hours of PDGF-AA and FGF-2. *Hmbs* was used as a reference gene; the reference gene *Eef1a1* showed similar results (data not shown). (b) Representative images of the centrosomic anchor (gamma-tubulin ( $\gamma$ -tub), red) with ARL13B (green) 3 days after initiation of differentiation. Note hardly any rod-like structures stained with ARL13B are observable. (c) Quantitative analysis of primary cilium display on scr- and *Ift88*-shRNA transduced gmOPCs (grey bars) and wmOPCs (white bars) 3 days after initiation of differentiation. Percentage of control cells displaying a primary cilium was  $45.4 \pm 12.7\%$  for gmOPCs and  $32.3 \pm 7.8\%$  for wmOPCs. (d,e) OPC proliferation of scr- and *Ift88*-shRNA transduced gmOPCs and wmOPCs was assessed by immunostaining of proliferation marker Ki67 (green) and the OPC

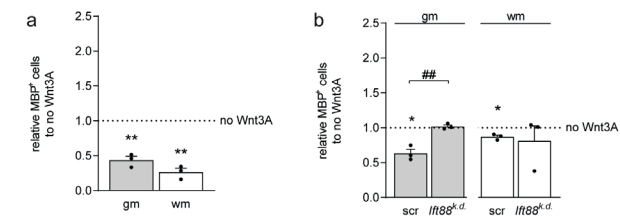
marker Olig2 (red) at 24 hours in the presence of the mitogens PDGF-AA and FGF-2. Representative images are shown in (d), quantification of the percentage of Ki67-positive cells of Olig2-positive cells in (e). The absolute percentage of Ki67-positive cells of Olig2-positive cells for ctrl was  $61.5 \pm 10.6\%$  for gmOPCs and  $69.0 \pm 8.9\%$  for wmOPCs. Note that the percentage of Ki67-positive cells is decreased in gmOPCs, but not wmOPCs, when *Ift88* is knocked down, and cilia are depleted. (f,g) OPC differentiation of scr- and *Ift88* shRNA-transduced gmOPCs and wmOPCs was assessed by immunostaining of differentiation marker MBP (red) at 6 days after initiating differentiation. Representative image of MBP staining shown in (f), quantification of the percentage of MBP-positive cells of DAPI-stained (blue) cells in (g). The absolute percentage of MBP-positive cells of DAPI-stained cells for ctrl was  $23.1 \pm 9.5\%$  for gmOPCs and  $15.6 \pm 2.7\%$  for wmOPCs. Note that the percentage of MBP-positive cells is decreased in gmOPCs, but not wmOPCs, when *Ift88* is knocked down and primary cilia depleted compared to scr. Bars represent relative means of *Ift88* expression (a), primary cilium display (c), Ki67-positive cells (d) and MBP-positive cells (e) in gmOPCs (grey bars) and wmOPCs (white bars) to control, which was set to 1 in each independent experiment (horizontal line). Error bars show the standard error of the mean. Statistical analyses are performed using a one-sample t-test to test for differences with control and an unpaired t-test was used to test between scramble and *Ift88<sup>kd</sup>*, indicated with # (\*#p<0.05, \*\*p<0.01). Scale bar is 50  $\mu$ m.

cells transiently increased at the immature OLG stage (Fig. 3d,e), while P2-derived gmOPCs harbor primary cilia earlier upon differentiation than P2-derived wmOPCs (Fig. 3d). As the appearance of primary cilia differed between differentiating P2-derived gmOPCs from P2-derived wmOPCs, the function of primary cilia on oligodendrocyte lineage cells was further examined with P2-derived OPCs.

### Primary cilia are involved in PDGF-AA- and FGF-2-mediated gmOPC proliferation, as well as gmOPC differentiation

OPC proliferation and differentiation are important parameters for successful remyelination<sup>3</sup>. To study the importance of primary cilia in regulating OPC behavior, we made use of a lentiviral short-hairpin RNA (shRNA) construct against *Ift88*. Primary cilia are unable to synthesize proteins, and therefore dependent on transport processes, regulated by intraflagellar transport proteins. *Ift88* encodes for intraflagellar transport protein 88 (IFT88), a protein that plays an essential role in primary ciliogenesis<sup>414</sup>. Lentiviral transduction with *Ift88* shRNA resulted in a 40-50% reduction in *Ift88* transcripts compared to non-transduced cells in both gmOPCs and wmOPCs (Fig. 4a, gmOPCs p=0.006 and wmOPCs p=0.047) and compared to scramble transduced OPCs (Fig. 4a, gmOPC p=0.067 and wmOPC p=0.012). Accordingly, the percentage of cells that display primary cilia was decreased by 60-75% in *Ift88*-shRNA-transduced OPCs (*Ift88<sup>kd</sup>*) compared to non-transduced cells (Fig. 4b,c, gmOPCs p=0.006 and wmOPCs p=0.001) and was more prominent

in gmOPCs than wmOPCs. Notably, the percentage of primary cilia-displaying cells was also reproducibly, but not significantly lower in scr-shRNA-transduced gmOPCs and wmOPCs (Fig. 4c). PDGF-AA and FGF-2 signaling may both initiate from the primary cilium<sup>403,404</sup> and are important interacting extracellular signaling cues for continued OPC proliferation<sup>415</sup>. Therefore, we next assessed PDGF-AA- and FGF-2-induced gmOPC and wmOPC proliferation upon the knockdown of primary cilia via *Ift88*. To assess OPC proliferation, OPCs were co-stained for Ki67, which stains actively proliferating cells<sup>416</sup>, and Olig2, 24 hours after PDGF-AA and FGF-2 exposure (Fig. 4d). After the knockdown of primary cilia via *Ift88*, a decrease in the percentage of Ki67-positive Olig2-positive gmOPCs, but not Olig2-positive wmOPCs was observed compared to non- and scr-transduced OPCs (Fig. 4e, gmOPCs, ctrl  $p=0.032$ , scr  $p=0.004$ ). Importantly, although PDGF-AA is a known ligand of the primary cilium<sup>417</sup> and PDGF-receptor  $\alpha$  (PDGFR $\alpha$ ) is a marker for OPCs<sup>80</sup>, and in line with a previous study<sup>59</sup>, PDGFR $\alpha$  did not colocalize with the primary cilium marker ARL13B in OPCs (Fig. S1). Given that the percentage of primary cilia-harboring cells transiently increase upon OPC differentiation, we next examined the effect of knockdown of primary cilia via *Ift88* on the percentage of MBP-positive cells, an indication for OPC differentiation (Fig. 4f). Of note, the defined culture medium for OPC differentiation contains signal factors, such as insulin, that may transduce their signal via primary cilia<sup>418</sup>. Both scr- and *Ift88* shRNA-transduced gmOPCs had a significant reduction in the percentage of MBP-positive cells after 6 days of differentiation (Fig. 4g, scr  $p=0.001$ , *Ift88*<sup>kd</sup>  $p=0.001$ ). The differentiation of *Ift88* shRNA-transduced gmOPCs was more reduced than scr-shRNA transduced cells (Fig. 4g,  $p=0.052$ ). In contrast, while the percentage of MBP-positive cells tended to decrease upon differentiation of *Ift88* shRNA-transduced wmOPCs, differentiation of scr-shRNA-transduced wmOPCs was reduced to the same extent (Fig. 4g). Hence, in addition to being involved in PDGF-AA and FGF-2-mediated gmOPC proliferation, but not wmOPC proliferation, the primary cilium may also be involved in regulating gmOPC and wmOPC differentiation. Being a signaling antenna for the cell, the primary cilium has been implicated in localized initiation of various signaling pathways (reviewed in <sup>402</sup>), including the regulation of non-canonical and canonical Wnt signaling<sup>398-401</sup>. As dysregulated canonical Wnt signaling interferes with OPC differentiation during developmental myelination and remyelination<sup>419,420</sup>, we next examined whether Wnt-mediated signaling in immature gmOLGs and wmOLGs proceeds via primary cilia.



**Figure 5.** Primary cilia are required for Wnt3a-mediated inhibition of immature grey matter, but not white matter oligodendrocyte differentiation. (a) Immature grey matter oligodendrocytes (gmOLGs, grey bar) and white matter OLGs (wmOLGs, white bar) were cultured in the presence of non-Wnt3a-enriched medium or Wnt3a-enriched medium for 3 days. Differentiation of immature gmOLGs and wmOLGs was assessed by immunostaining of differentiation marker MBP. Note that Wnt3a-enriched medium exposure decreases the percentage of MBP-positive gmOLGs and wmOLGs. The absolute percentage of MBP-positive cells of DAPI-stained cells for non-Wnt3a-treated gmOLGs  $9.9 \pm 3.9\%$  gmOLGs and wmOLGs  $11.6 \pm 2.4\%$ . (b) *Ift88*, essential for primary cilium formation, was knocked down by short-hairpin RNA interference in mixed glial cultures from cortex (GM) and non-cortex (WM) obtained from newborn rats (postnatal day 2). Oligodendrocyte progenitor cells (OPCs) were shaken off and allowed to differentiate for 3 days to imOLGs. ImOLGs were cultured in the presence of non-Wnt3a-enriched medium or Wnt3a-enriched medium for 3 days and differentiation assessed by immunostaining for MBP. Cells transduced with scrambled (scr) shRNA were used as control. Note that Wnt3a-enriched medium inhibits differentiation in control and scr shRNA-transduced cells, but not in cells in which *Ift88* was knocked down and primary cilia depleted in immature gmOLGs, but not wmOLGs. The absolute percentage of MBP-positive cells of DAPI-stained cells was for non-Wnt3a medium treated scr shRNA-transduced immature gmOLGs  $18.3 \pm 11.9\%$ , immature wmOLGs  $10.5 \pm 5.0\%$ , *Ift88*<sup>kd</sup> immature gmOLGs  $8.0 \pm 4.0\%$  and immature wmOLGs  $6.3 \pm 1.8\%$ . Bars represent relative means of MBP expression to control (a) or non-Wnt3a-enriched medium-treated respective controls (b), which was set to 1 in each independent experiment (horizontal line). Grey bars represent gmOPCs, white bars represent wmOPCs. Error bars show the standard error of the mean. Statistical analyses were performed using a one-sample t-test to compare differences between treatment and control, indicated with \*, and an unpaired t-test was used to test between scramble and *Ift88*<sup>kd</sup>, indicated with # (\*# $p<0.05$ , \*\*/### $p<0.01$ , \*\*\* $p<0.001$ ).

### The primary cilium is required for Wnt3a-mediated inhibition of immature grey matter, but not white matter oligodendrocyte differentiation

Previous findings revealed that exposure to Wnt3a, a Wnt signaling agonist, has opposite differentiation-stage effects on OPC maturation. Thus, while addition of Wnt3a prevents early OPC differentiation<sup>59,420</sup>, Wnt3a signaling in more mature OLGs is required for myelinogenesis<sup>421</sup>. Therefore, we first assessed the effect of Wnt3a signaling on the differentiation of immature OLGs, i.e., three days after initiating differentiation when most cells display a primary cilium (Fig. 3). To this



end, immature gmOLGs and wmOLGs were exposed to Wnt3a-enriched medium for three days followed by MBP immunocytochemistry. A prominent and significant decrease in the percentage of MBP-positive cells of Wnt3a-exposed immature gmOLGs and wmOLGs was observed compared to immature gmOLGs and wmOLGs that were exposed to medium that was not enriched for Wnt3a (Fig. 5a, immature gmOLGs  $p=0.009$  immature wmOLGs  $p=0.005$ ). To examine whether the presence of a primary cilium is essential for Wnt3a-mediated inhibition of imOLG differentiation, the effect of Wnt3a on immature gmOLG and wmOLG differentiation was assessed in scr-shRNA- and *Ift88*-shRNA-transduced cells. The differentiation-inhibiting effect of Wnt3a-enriched medium compared to medium that was not enriched for Wnt3a, was also observed in scr-shRNA-transduced immature gmOLGs and wmOLGs (Fig. 5b,  $p=0.026$  for gmOLGs,  $p=0.033$  for wmOLGs), but not in *Ift88*-shRNA transduced-immature gmOLGs and wmOLGs. Notably, exposure to Wnt3a-enriched medium significantly decreased the percentage of MBP-positive cells of scr-shRNA-transduced gmOLGs compared to *Ift88*-shRNA-transduced immature gmOLGs (Fig. 5b,  $p=0.004$ ), while the percentage of MBP-positive cells did not differ between *Ift88*-shRNA-transduced and scr-shRNA-transduced immature wmOLGs (Fig. 5b). Hence, initiation of Wnt3a-signaling via the primary cilium appeared required for the inhibition of immature gmOLG differentiation but not immature wmOLG differentiation.

## Discussion

Remyelination is more robust following demyelination of the GM compared to the WM, both in MS<sup>26,27,353</sup> and upon cuprizone-induced demyelination<sup>25,244</sup>. Regional differences in remyelination efficiency may be a reflection of regional diversity in OPCs<sup>37-39,41,44,138,144</sup>, including a distinct responsiveness to environmental signals<sup>44,144</sup>. Here, we explored whether the display and function of the primary cilium, a cell signaling hub, differed between oligodendrocyte lineage cells of the GM and WM. Our findings revealed that at conditions of active myelination, i.e., in the neonatal brain, upon demyelination and upon *in vitro* OPC differentiation, more GM oligodendrocyte lineage cells displayed a primary cilium than WM oligodendrocyte lineage cells. In addition, PDGF-AA and FGF-2-induced gmOPC proliferation and Wnt3a-mediated inhibition of immature gmOLG differentiation depended on the presence of primary cilia, while primary cilia were dispensable for the regulation of these processes in wmOPCs and immature wmOLGs. As primary cilia act as sensory and coordinating signaling hubs, gmOPCs may respond more rapidly and/or distinctly to temporally changes in environment, such as demyelinating injury, than wmOPCs, which may contribute to the regional differences in remyelination efficiency.

Primary cilia are dynamic organelles enriched in signaling receptors that can be assembled and disassembled in response to environmental cues, including injury. Our data indicate that the dynamics of primary cilia formation on oligodendrocyte lineage cells differed in cortex and corpus callosum at stages of active myelination. At P7, but not at P2, a higher percentage of oligodendrocyte lineage cells displayed primary cilia in the cortex than in the corpus callosum. A similar tendency in a transient increase in the percentage primary cilia-displaying oligodendrocyte lineage cells in the cortex but not corpus callosum was observed upon cuprizone-induced demyelination. Of note, although a minority of oligodendrocyte lineage cells, which comprise in the neonatal brain and demyelinated areas mainly OPCs and immature OLGs, display a primary cilium, given its transient increase, this may be an underestimation. In line with a temporary display, was the transient appearance of a primary cilium on both differentiating gmOPCs and wmOPCs, reaching a maximum at the immature OLG stage, i.e., at the onset of myelin membrane formation. Upon early differentiation *in vitro*, more gmOPCs harbor a primary cilium than wmOPCs. As primary wmOPCs

are more mature and differentiate faster<sup>144</sup>, it cannot be excluded that the difference in the percentage of primary cilia-displaying oligodendrocyte lineage cells between cortex and corpus callosum at P7 and differentiating gmOPCs and wmOPCs reflect a difference in the timing of primary cilium formation as of a regional difference in maturation *in vitro* and *in vivo*<sup>144,422</sup>. This is less likely in the demyelinated areas, as remyelination proceeds faster in the cortex than in the corpus callosum<sup>25</sup>. Hence, our findings indicate that upon *in vitro* differentiation of gmOPCs and wmOPCs primary cilia were transiently assembled at immature OLGs, while *in vivo* an increase in primary cilia-displaying oligodendrocyte lineage cells was mainly observed in the cortex.

The primary cilium although continuous with the plasma membrane is a dynamic organelle that organizes and concentrates spatial-temporal signaling networks and the antenna enables cells to detect changes in the environment. Therefore, a differential display or function of primary cilia on oligodendrocyte lineage cells may have consequences for the regulation of signaling processes and cell behavior. Indeed, disruption of primary cilia ciliogenesis by a knock down of *Ift88* partially reduced PDGF-AA and FGF-2-induced gmOPC, but not wmOPC proliferation. Notably, we have previously shown that *in vitro* gmOPCs proliferate more in response to PDGF-AA and FGF-2 than wmOPCs<sup>144</sup>. A role for primary cilia in PDGF-induced gmOPC proliferation has been previously demonstrated, which involved Shh-mediated signaling at the primary cilium, but not PDGF-mediated signaling, given the absence of PDGFR $\alpha$  at primary cilia<sup>59</sup>. Our findings elaborate on these findings and demonstrate that also that in the absence of Shh, OPC proliferation is at least partially mediated via the primary cilium in gmOPCs. Notably, FGF receptors have been associated with the primary cilium<sup>404</sup>, and FGF signaling may partially rely on signal transduction events in the primary cilium in gmOPCs. A putative role of the primary cilium on OPC proliferation in the developing and demyelinated cortex in coordinating or amplifying of signal transduction pathways of distinct mitogens involved in OPC proliferation remains to be determined.

Our findings further revealed that addition of Wnt3a, an agonist for Wnt-signaling, to primary cultures had an inhibitory effect both on immature gmOLG and wmOLG

differentiation. The presence of primary cilia was indispensable for Wnt3a-mediated inhibition of immature gmOLG differentiation, but not immature wmOLG differentiation, indicating that the initiation of Wnt3a signaling is differentially regulated between immature gmOLG and wmOLG differentiation. Canonical Wnt signaling, including Wnt ligands, frizzled receptors,  $\beta$ -catenin, and Tcf transcription factors has, depending on developmental stage, region and dose, beneficial and detrimental roles during the myelinating process (reviewed in <sup>423</sup>). More specifically, Wnt/ $\beta$ -catenin signaling inhibits gmOPC and wmOPC specification<sup>419</sup>, prevents gmOPC and wmOPC differentiation<sup>59,420</sup>, and increases myelin protein expression in mature OLGs from spinal cord<sup>421</sup>. Whether Wnt signaling in these processes also proceeds via primary cilia is unexplored. Wnt signaling can also proceed outside the primary cilium, and signaling at the primary cilia may constitute different signaling events that distinctly or similarly affect cellular processes. In fact, the role of primary cilia in Wnt signaling is diverse (reviewed in <sup>402</sup>) with studies supporting a role of primary cilia or cilia-related proteins in promoting or restraining Wnt signaling<sup>398-401</sup>, from finding no role<sup>424</sup> to Wnt signaling being involved in primary assembly and disassembly<sup>425</sup>. Hence, temporal-specific initiation and regulation of the Wnt-signaling pathway likely depends on differentiation state, region and the presence of primary cilia, which may have consequences for converged signaling pathways with other environmental cues, such as BMP<sup>426</sup>, in regulating OPC maturation.

Taken together, our data indicate that during active myelination, the regulation of OPC proliferation and differentiation in the GM, but not WM, may rely on receiving and processing signals at the primary cilia. Developmental myelination and remyelination are tightly regulated processes that depend on sequential appearance and interacting signaling cues<sup>3</sup>. Partially organizing these events in primary cilia may alter the responsiveness to environmental signaling cues and/or fine-tune signaling processes. This may contribute to more efficient remyelination in GM than in WM. Following this reasoning, it would be interesting to examine whether OPCs in GM and WM MS lesions harbor functional primary cilia, and whether their potential absence or dysfunction is linked to remyelination failure and disease progression. OPCs in MS lesions may either not assemble or lose their primary cilium in response to environmental cues. Hence, both elucidation of ligands and

the signaling pathways that initiate at the primary cilium that are involved in OPC behavior at physiological conditions and disease, and understanding the regulation of primary ciliogenesis in OPCs, may not only add to our understanding of regional differences in remyelination efficiency but also its failure.

## Methods

### *Animals*

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (the Netherlands). All methods were carried out according to national and local experimental animal guidelines and regulations.

### *Rat brain tissue*

Brains were dissected from newborn Wistar rats (Envigo) at postnatal days 2 (P2) and 7 (P7), immediately snap-frozen in liquid nitrogen, and stored at -80°C until further use.

### *Cuprizone-induced demyelination*

Demyelination was induced in 8-10 week-old individually housed adult male C57Bl/6 mice (Harlan) by supplementation of 0.2% cuprizone (bis-cyclohexanone oxalhydrozone, Sigma) in standard rodent chow for 5 weeks. Remyelination was induced by switching to normal chow for 2 weeks. Animals were sacrificed and perfused with 4% paraformaldehyde (PFA) after 3 (demyelinating) or 5 weeks (demyelinated and early signs of remyelination) of cuprizone and after 2 weeks of remyelination (remyelinated), followed by 20% sucrose in phosphate buffered saline (PBS) overnight. Brains were stored at -80°C until further use. Demyelination and remyelination was confirmed by Sudan black staining (0.1% in 70% ethanol for 5 minutes). Control animals were sacrificed at 15-17 weeks of age.

### *Primary cultures*

Enriched OPC cultures were obtained from newborn Wistar rat brains (Po-2 or P7, Envigo), as described<sup>144</sup>. Briefly, mixed glial cultures from the neonatal rat cortex (referred to as gmOPCs) and neonatal non-cortical parts (white matter (WM) tracts including corpus callosum, mixed grey matter (GM) and WM tracts, including hippocampus and thalamus, and deep GM parts, including basal ganglia, referred to as wmOPCs) were cultured for 12 days after which OPCs were obtained via a shaken

off procedure. This results in >95% Olig2-positive P2 and P7 OPC cultures (Olig2-positive). OPCs were cultured in defined Sato medium<sup>144</sup> on poly-L-lysine-coated (5 µg/ml) 13-mm glass coverslips in 24-well plates (immunocytochemistry) at a density of  $3 \times 10^4$  and  $4 \times 10^4$  for gmOPCs and wmOPCs respectively or 10 cm dishes (qPCR) at a density of  $10^6$  cells. One hour after plating 10 ng/ml platelet-derived growth factor-AA (PDGF-AA; Peprotech, cat. no. 100-13) and 10 ng/ml human fibroblast growth factor-2 (FGF-2; Peprotech, cat. no. 100-18) were added for 48 hours to synchronize OPCs to the early bipolar stage. OPC differentiation was induced by replacing growth factor-containing medium for Sato medium supplemented with 0.5% non-heat inactivated fetal bovine serum (FBS, Capricorn Scientific, cat. no. FBS-12A) for the indicated days. At day 3 of differentiation, cells are considered immature and at 6 days of differentiation mature OLGs. Wnt3a-enriched Sato medium was added from day 3 to day 6 where indicated. For lentiviral transduction, prior to the shake off, P2 or P7 mixed glial cultures were exposed to viral particles (1:4 in mixed glial medium) in the presence of polybrene (8 µg/ml, Sigma) for 16 hours.

#### *Wnt3a-enriched medium*

Wnt3a-enriched medium was a kind gift from the Coppes lab (University Medical Center Groningen, Groningen, the Netherlands). In brief, the L Wnt-3A cell line was cultured in growing medium (10% FBS (Sigma, cat. no. F7524) and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, cat. no. 15140) in DMEM (Life Technologies cat. no. 31966)). Growing medium was harvested after 1 week, centrifuged for 5 minutes at 1500 rpm and filtered through a 0.22 µm filter (Millipore, cat. no. SLGV033R). The filtered medium was then used to produce Wnt3a-enriched Sato medium (50% filtered WNT3A enriched medium, 10% R-spondin-conditioned medium (AMSBIO, RSPO1 cell line (provided by Dr. C. Kuo (Stanford University School of Medicine, Stanford, CA)), 40% 2x Sato<sup>144</sup>). Medium not enriched in WNT3A (non-enriched medium) was produced in parallel using a control cell line.

#### **Lentivirus production and transduction**

Primary cilia ciliogenesis in OPCs was downregulated by lentiviral transduction of *Ift88* specific shRNA. To this end, lentiviral particles were created using the constructs

pLKO.1-puro Sigma mission shRNA, construct SHCLNG-NM\_009376, sequence TRCN0000178064, for *Ift88* shRNA, or SHC002 for control scrambled shRNA; 5 µg), envelope and packaging plasmids (500ng pMD2-VSVG; 5 µg pCMV-R8.91) were transfected using Fugene (Promega, E2311) into the HEK293T cell line according to manufacturer's instructions. After 16 hours, medium was changed to mixed glial culture medium<sup>144</sup>. After 24 hours, viral particles were harvested and new mixed glial culture medium was added to the HEK293T cells. After another 24 hours, viral particles were harvested and combined with the first batch and filtered through a 0.45 µm filter (GE healthcare). Viral particles were stored at -80°C until further use.

#### **Immunohistochemistry**

Non-fixed, snap-frozen brains were sectioned at 16 µm and stored at -80°C until further use. Slices were exsiccated for 30 minutes and fixed for 15 minutes using 4% PFA. Antigens were retrieved by heating in citrate buffer (pH 6.0). Non-specific antibody binding was blocked using 5% normal goat serum, 3% FBS and in 0.1% Triton-X100 in PBS for 30 minutes. Slices were incubated overnight at 4°C with primary antibodies against ARL13B and Olig2 (listed in table S1), followed by incubation with the appropriate secondary Alexa-conjugated antibodies (1:500, Thermo Fisher Scientific) for 1 hour at room temperature (RT). Nuclei were stained using Hoechst (Sigma, cat. no. 33342; 1 µg/ml in PBS) for 5 minutes. Slices were mounted in Mowioil. In each independent experiment, >150 Olig2-positive-cells were scored per region and condition in a blinded manner at approximately Bregma -1.0 throughout the whole corpus callosum and cortex, excluding the piriform cortex. Analysis was performed using a conventional immunofluorescence microscope (Leica DMI6000 B with Leica Application Suite Advanced Fluorescence software).

#### **Immunocytochemistry**

Immunolabelling using the antibody A2B5 was performed at 4°C on living cells. Non-specific antibody binding was blocked using 4% bovine serum albumin (BSA) for 10 minutes. Cells were rinsed twice with ice-cold PBS and incubated with antibody A2B5 (1:5 in 4% BSA) for 30 minutes. Cells were then incubated with the appropriate Alexa-conjugated antibodies for 20 minutes and fixed using 4% PFA in

PBS for 20 minutes at RT. For intracellular immunostaining, 4% PFA-fixed cells were permeabilized with ice-cold methanol for 10 minutes. Non-specific antibody binding was blocked with 4% BSA for 30 minutes after which cells were incubated with antibodies against ARL13B, Ki67, MBP, Olig2, PDGFR $\alpha$  or  $\gamma$ -tub (table S1) at RT. Cells were washed three times with PBS before the appropriate FITC-/TRITC-conjugated antibodies (1:50, Jackson ImmunoResearch) were added for 30 minutes at RT. Nuclei were visualized using 4',6-diamidino-2-phenylindole ([DAPI], 1mg/mL, Sigma). After washing three times in PBS, cells were mounted in DAKO mounting medium, and analyzed using a conventional immunofluorescence microscope (Leica DMI 6000 B with Leica Application Suite Advanced Fluorescence software). In each independent experiment, >150 cells were scored per condition. Proliferation was defined by the percentage of Ki67-positive cells of Olig2-stained cells. OPC differentiation was defined as the percentage of MBP-positive cells of total DAPI-stained cells.

### qPCR analysis

Cells were gently scraped in RNAlater (Qiagen, cat. no. 76526) and centrifuged at 10 000rpm for 7.5 minutes at 4°C. mRNA was extracted (Isolate II RNA Micro Kit; Bioline, cat. no. BIO-52075) and 100 ng total RNA was reverse transcribed in the presence of oligo(dT)<sub>12-18</sub> (Invitrogen, cat. no. 18418012) and dNTPs (Invitrogen, cat. no. 10297018) with M-MLV reverse transcriptase (Invitrogen, cat. no. 28025013) according to manufacturer's instructions. Gene expression levels were quantified by real-time quantitative RT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad, cat. no. 172-5124). Measurements were performed in triplicate and data were processed using the LinRegPCR method<sup>391,392</sup>. Primer sequences are listed in table S2.

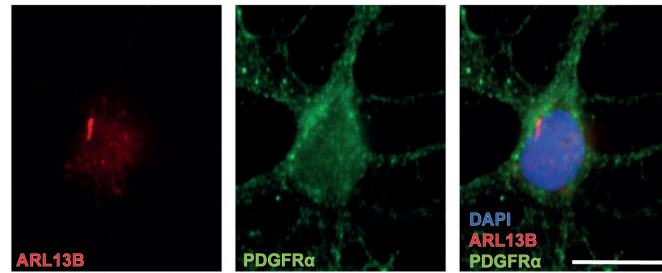
### Statistical analyses

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 6.0. A one-sample t-test was used when relative values were compared with control, which was set to 1 in each independent experiment. A two-sided paired t-test was used to compare gmOPCs and wmOPCs, a two-sided, unpaired t-test was used to compare between scramble and *Ift88*<sup>k.d.</sup>, and a one-way ANOVA with a Dunnett post-test to compare between multiple groups and

their control when control values were not set to 1, or with a Tukey's post-test when comparing between multiple treatment groups. P-values of <0.05, <0.01, and <0.001 were considered significant and indicated with \*, \*\*, \*\*\* respectively.

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**Figure S1.** PDGFR $\alpha$  hardly co-localize with primary cilia on oligodendrocyte progenitor cells. Oligodendrocyte progenitor cells (OPCs) isolated from postnatal day 2 brain cortex (gmOPCs) were cultured for 48 hours in the presence of mitogens PDGF-AA and FGF-2. The location of PDGFR $\alpha$  (green) in respect to the primary cilium (ARL13B, red) and the cell nucleus (DAPI, blue) was visualized by immunocytochemistry. Similar findings were observed for wmOPCs. Note that PDGFR $\alpha$  is not localized at the primary cilium. Scale bar is 50  $\mu$ m.