Wnt-Dependent Oligodendroglial-Endothelial Interactions Regulate White Matter Vascularization and Attenuate Injury

Graphical Abstract

Highlights

- OPC density promotes white matter vascularization through tip cell angiogenesis
- Hypoxic OPCs in human HIE lesions activate canonical Wnt pathway in endothelium
- OPC-Wntless cKO caused disrupted tip cell angiogenesis and myelination deficits
- OPC-Wnt7a/7b double-KO mice display increased susceptibility to hypoxic injury

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In Brief

Chavali et al. demonstrate that oligodendroglial precursor density and their interactions with endothelial tip cells regulate white matter vascular development. In hypoxic brain injury, OPCs activate canonical Wnt signaling in angiogenic endothelial cells. Ablation of OPC-derived Wnt7 ligands results in disrupted white matter tip-cell angiogenesis and myelination defects.
Wnt-Dependent Oligodendroglial-Endothelial Interactions Regulate White Matter Vascularization and Attenuate Injury

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SUMMARY

Recent studies have indicated oligodendroglial-vascular crosstalk during brain development, but the underlying mechanisms are incompletely understood. We report that oligodendrocyte precursor cells (OPCs) contact sprouting endothelial tip cells in mouse, ferret, and human neonatal white matter. Using transgenic mice, we show that increased or decreased OPC density results in cognate changes in white matter vascular investment. Hypoxia induced increases in OPC numbers, vessel density and endothelial cell expression of the Wnt pathway targets Apcdd1 and Axin2 in white matter, suggesting paracrine OPC-endothelial signaling. Conditional knockout of OPC Wntless resulted in diminished white matter vascular growth in normoxia, whereas loss of Wnt7a/b function blunted the angiogenic response to hypoxia, resulting in severe white matter damage. These findings indicate that OPC-endothelial cell interactions regulate neonatal white matter vascular development in a Wnt-dependent manner and further suggest this mechanism is important in attenuating hypoxic injury.

INTRODUCTION

Oligodendrocytes (OLs) are the myelinating cells of the central nervous system. During the process of myelination, developing oligodendrocyte precursors (OPCs) undergo dramatic changes in morphology and size as they differentiate, in some cases, achieving a 7,000-fold increase in membrane volume, to supply hundreds of myelin segments for nerve axons (Baron and Hoekstra, 2010; Webster, 1971). The consequent bioenergetic requirements imply that the OPCs must have adequate access to a vascular network for nutrients, oxidative and metabolic substrates during myelination. Indeed, oligodendroglia intimately associate with the developing vasculature (Tsai et al., 2016; Yuen et al., 2014), and endothelial cells (ECs) have been shown to regulate myelination in development and disease (Niu et al., 2019; Rajani et al., 2018; Swire et al., 2019), suggesting bi-directional cell-cell interactions.

In the mammalian brain, angiogenesis commences at embryonic day 9 (E9), when superficial pial blood vessels (BV) ingress into the deeper parenchyma (Greenberg and Jin, 2005; Plate, 1999; Vasudevan et al., 2008). By E10, a ventral periventricular plexus forms and starts to branch into the dorsal telencephalon to join the pial vessels (Vasudevan et al., 2008). This network is refined via EC proliferation, sprouting, branching, vessel regression and stabilization (Carmeliet and Jain, 2011), processes regulated by local secretion of vascular endothelial growth factors (VEGFs), bone morphogenetic proteins (BMPs), Wnts, and axon guidance cues derived from various neural cell types (Adams and Eichmann, 2010; Carmeliet and Jain, 2011; Eidmann and Thomas, 2013). For example, neuroepithelial Wnt7a/b function has been shown to be essential for embryonic CNS vascular development (Cho et al., 2017; Daneman et al., 2009; Stenman et al., 2008; Zhou et al., 2014).

The embryonic vasculature undergoes further remodeling in the postnatal and adult stages in a region-dependent manner (Bozoyan et al., 2012; Harb et al., 2013). In this context, neuronal reelin regulates cortical vascular organization through activation of endothelial intrinsic Disabled 1 (Dab1) and Apolipoprotein E receptor 2 (ApoER2) signaling; disruption in this process results in neuronal positioning errors (Segarra et al., 2018). Adult neurogenesis and angiogenesis are also coupled...
in the germinal centers of brain, the subventricular zone and the subgranular zones (Le Magueresse et al., 2012; Shen et al., 2019; Tavazoie et al., 2008; Wang et al., 2019), where vascular-neural-cell crosstalk regulates neuronal progeny generation and migration.

Here, we show that OPCs physically interact with angiogenic tip cells in developing white matter (WM). Previous studies have indicated regional differences in regulation of vascular development coupled to metabolic activity and myelination (Harris and Attwell, 2012; Lam et al., 2010; Paredes et al., 2018). We first investigated the possibility that OPCs physically interact with sprouting endothelial tip cells in WM. As shown (Figure 1A), such physical co-location was apparent through immunohistochemical analysis of P1 neonatal mouse brains with markers for OPCs (PDGFRα+Olig2+) and ECs (CD31+). Isosurface reconstruction shows OPC processes enwrap tip cell filopodia (white arrowheads). Scale bars: 25 µm.

(B and C) Representative images and isosurface reconstructions from a P1 Sox10-GFP mouse showing CD31+ tip cell filopodia are frequently enwrapped by complex GFP+ OPC processes (white arrowheads) but not astroglial (GFAP+) processes. Scale bar: 20 µm.

(D) Quantification of tip cells contacted by OPCs and astroglial cells or both in the P1 WM. (E and E’) Representative images from P10 ferret brain coronal section and isosurface reconstruction showing OPCs in the WM frequently enwrap endothelial tip cell filopodia. Scale bars: 25 µm.

(F and F’) OPC-tip-cell interactions in 17 gestational week (GW) human brain coronal section, showing similar findings to mouse and ferret. Scale bars: 25 µm.

(G and G’) Persistent oligodendroglial-endothelial tip cell interactions shown by ultrastructural analysis of subcortical WM resection from a 2-year-old human, combined with Olig2 immunogold labeling. Note oligodendroglial cell process (pseudocolor-green; black arrowhead) contacting filopodial extensions from a nascent vessel (pseudocolor-red) with caveolae (black arrows). Scale bars: 5 µm (G), 500 nm (G’).

BV, blood vessel; RBC, red blood cell; O, oligodendroglia. Data are represented as means ± SD and are analyzed with a two-tailed unpaired Student’s t test. n = 3 animals. *p < 0.05, **p < 0.01.
1E, 1F, and S1B–S1D). Because angiogenic sprouting continues until the second postnatal week in mice (Figure S1E), we investigated such a conservation in human brain by ultrastructural analysis of resected subcortical WM tissue from a 2-year-old human combined with Olig2 immunogold labeling (Figure 1G). We found that oligodendroglial cell (Olig2 immunogold) processes formed similar physical contacts with nascent vessels with increased caveolae (Figure 1G and 1G'). The cell membrane at the OPC contact site was characterized by the lack of a basement membrane, indicative of tip cell filopodia (Figure 1G'). These results suggest that physical interactions of OPCs and endothelial tip cells occur during the neonatal and postnatal WM development, a period during which active vascular network formation occurs.

### Decreased OPC Density Is Associated with WM Hypovascularization

Because we observed frequent OPC-endothelial-tip-cell interactions (Figure 2A), we asked whether those interactions had a functional significance on WM vascular development. To that end, we intercrossed several lines of transgenic mice to alter OPC numbers in the developing brain. To deplete OPC numbers, we generated Olig2-cre, Sox10-lox-GFP-STOP-lox-DTA (hereafter, called Sox10-DTA), a transgenic line in which diphtheria toxin fragment A (DTA) falls under the transcriptional control of the Sox10 promoter, with toxic expression restricted to OPCs (Figures 2B, 2A, and 2B) (Kessaris et al., 2006). As shown in Figures 2C–2E, we found an ~65% decrease in Olig2+ cell density in WM and cortical regions of Sox10-DTA mice at P1 compared with controls. Interestingly, we observed that WM vascular network was hypoplastic at that time point (Figures 2C–2F).

Although the Olig2-Cre line targeted up to 75% of OPCs in the neonatal WM (Figure S2A), the cells that escaped Cre-targeting subsequently started to repopulate the brain (Figures S2B and S2C), and by P11, Olig2+ cell numbers had increased by ~15% (Figures 2G–2I and S2D). We, therefore, assessed the effect of OPC recovery on WM vascular development. As shown (Figures 2G–2J), although there was a slight increase in Olig2+ cell numbers at P11, the vessel density, length, and branching remained significantly lower than controls. Despite that, we did not detect abnormalities in the blood-brain-barrier (BBB) structure or permeability, the astrocyte endfeet coverage, or the microglial inflammatory response (Figures S2E–S2H). These findings indicated a positive correlation between oligodendroglial numbers and vascular density in WM (Figure 2K).

### Increased Oligodendroglial Density Results in WM-Specific Hypervascularization

We next tested whether we could promote WM hypervascularization by bolstering OPC numbers. To achieve that, we intercrossed Olig2-Cre and BrafV600E(fl/+)- mice (Dankort et al., 2009) (constitutively active B-Raf; murine sarcoma viral oncogene homolog B, termed BRAFCA hereafter) (Figure 3A). Overactivation of B-Raf signaling is a mitogenic driver of OPCs (Huillard et al., 2012). Analysis of BRAFCA mice at P0 and P9 indicated a significant increase in Olig2+ cell numbers and vessel density, branching length in the WM (Figures 3B–3I); this phenotype was sustained until P14, the extent of animal survival (Figures S3A–S3C). A recent study showed that tight perivascular clustering of OPCs on BVs in pathological conditions results in BBB damage (Niu et al., 2019). Although OPCs in BRAFCA mice did cluster on the vessels in the WM (Figure 3J), we did not observe any disruptions in astrocyte endfeet coverage or BBB damage, as assessed by unaltered PLVAP and Glut1/Claudin 5 expression in vessels and, importantly, an absence of low-molecular-weight cadaverine leakage into brain parenchyma (Figures 3K–3N and S3D). Together, these results indicated that increased OPC numbers directly correlated with the density of the WM vascular network (Figure 3O).

To determine whether OPC density also affected BV coverage in the gray matter, we analyzed the motor and somatosensory cortex from Sox10-DTA and BRAFCA animals. Although the oligodendroglial cell density was significantly decreased at both the P1 and P11 in Sox10-DTA cortex, no corresponding decreases in vascular density were noted in those regions (Figures S4A–S4C). Similarly, although oligodendroglial cell density was significantly increased in BRAFCA cortex at both P0 and P9, we did not detect any vascular density increase in those regions (Figures S4D–S4F). These results indicate that the oligodendroglial-density-induced vascular network formation is WM specific.

### Chronic Hypoxic Injury Acutely Increases OPC Density and WM Vascular Investment in Ferret and Human Neonatal Brain

To assess the pathophysiological relevance of OPC-vascular interactions in WM, we first tested a ferret chronic sublethal hypoxia model (Figure 4A). As shown (Figure 4B), chronic exposure of neonatal kits to mild hypoxia (10% FIO2) from P10 to P20 resulted in hypomyelination consistent with previous reports (Tao et al., 2012). We found that hypoxia caused a significant increase in the numbers of immature PDGFRα+Olig2+ OPCs in WM tracts with reduced numbers of immature BCAS1+Olig2+ premyelinating cells (Fard et al., 2017) (Figures 4C, 4D, and 4F). Interestingly, as shown (Figures 4E and 4F), we also observed a significant increase in vascular density accompanied by greater vessel length and branching in the WM tracts of hypoxia-reared ferret kits.

Human neonatal hypoxic-ischemic encephalopathy (HIE) causes neuronal cell death, gliosis, and WM injury (Figure S5A) (Billiards et al., 2008; Kinney and Back, 1998; Liu and McCulloch, 2013; Northington et al., 2011). We analyzed WM tracts from six cases of HIE and age-matched controls (Figure 4G; Table S1). Intriguingly, histological analysis of vascular markers showed a significantly increased vessel density and branching in the WM tracts of the HIE cases (Figures 4H and 4I). Furthermore, we also found an increase in OPCs (PDGFRα+Olig2+) (Figures 4J and 4M) and reduced numbers of both premyelinating (BCAS1+Olig2+) and myelinating (Nogo-A+Olig2+) OLs in the HIE (Figures 4K–4M). We found no alterations in vascular junctional marker Claudin 5, indicating vascular integrity was not disrupted (Figure S5B). Together, these results indicate increased WM OPC and vascular densities in human HIE (Figure 4N).
Endothelial Wnt Signaling Targets Gene Upregulation in Human HIE

Histological analysis of human neonatal brain revealed that OPC proximity to BVs coincided with increased expression of the Wnt signaling downstream target Lef1 in ECs (Figure S5C). Because OPCs make physical contacts with angiogenic vessels and given that Wnts are short-range signals that can induce downstream activation in neighboring cells (Figure 4O) (Clevers et al., 2014), we further assessed human HIE cases and controls for evidence of active OPC-driven Wnt signaling in ECs by immunohistochemistry and single-molecule fluorescence in situ hybridization (smFISH). As shown (Figures 4P, S5D, and S5E), we found that OPCs expressed WNT7A mRNA, encoding an angiogenic Wnt, in HIE lesions. We next assessed expression of the Wnt transcriptional targets APCDD1 and AXIN2 in CD31+ ECs in HIE lesions and observed a significant increase in their transcripts (Figures 4Q and 4R). In line with those results, we also observed an increase in Lef1+ ECs in WM tracts of HIE cases as well as in

Figure 2. OPC Ablation Leads to Developmental Hypovascularization of White Matter in Sox10-DTA Transgenic Mice

(A) Cartoon of neuronal and glial cell interactions with developing vasculature in the neonatal forebrain.
(B) Genetic strategy for Sox10-DTA mouse generation.
(C) Representative images of P1 mouse brain coronal sections labeled with CD31 showing significant decrease in WM vessel density in Sox10-DTA mouse compared to control (CTRL). Scale bar: 100 μm.
(D) High-magnification images of the cingulum region, highlighted areas from (C), showing Sox10-GFP+/Olig2+ oligodendroglial cells and CD31+ BVs. Scale bar: 50 μm.
(E) Regions of WM analyzed along the rostro-caudal axis.
(E') Quantification of Olig2+ cells in the P1 WM.
(F) Quantification of BV coverage, branching, and length in the P1 WM.
(G) Representative images from P11 mouse brain coronal sections labeled with CD31 showing significant decrease in WM vessel density in Sox10-DTA mouse compared to CTRL. Scale bar: 100 μm.
(H) High-magnification images of the cingulum region, highlighted areas from (G), showing Sox10-GFP+/Olig2+ oligodendroglial cells and CD31+ BVs. Scale bar: 50 μm.
(I) Quantification of Olig2+ cells in the P11 WM.
(J) Quantification of BV coverage, branching, and length in the P11 WM.
(K) Oligodendroglial cell density and vascular coverage in WM are positively correlated.

Data are represented as means ± SD and are analyzed with a two-tailed unpaired Student’s t test. n = 6 animals/genotype (E' and F); n = 5 (I–K). *p < 0.05, **p < 0.01, ***p < 0.0001.
hypoxic ferret brain (Figures S5F–S5H). Together, these findings suggest that Wnt ligand activity in human OPCs results in paracrine activation of Wnt/β-catenin target gene activation in ECs of WM affected by hypoxic injury.

OPC-Encoded Wntless Function Is Essential for Neonatal WM Vessel Development

Because increased WM vascular density in human HIE lesions was coupled with Wnt target gene expression in endothelia, we next asked whether OPC-encoded Wnt ligands have a functional role in WM vascular development in vivo. To that end, we generated an OPC conditional knockout of Wntless (Carpenter et al., 2010), a transmembrane protein required for Wnt family trafficking and secretion (Banziger et al., 2006) (Figures 5A and 5B). At P3, conditional knockout of Wntless in OPCs (hereafter, Wntless cKO) did not result in WM abnormalities (Figures 5C, 5D, and 5E); however, by P7, the WM vascular density started to deteriorate and was significantly reduced by P12 (Figures 5E–5J). Oligodendroglial cell numbers remained unaltered at P7 in Wntless cKO WM, but their numbers declined significantly by P12 (Figures 5E, 5F, 5H, and 5I). These findings indicated a functional requirement for OPC-derived Wnt cues.

Canonical Wnt signaling is essential for BBB maturation (Danneman et al., 2009; Stenman et al., 2008). To understand whether Wnt production from OPCs is needed for BBB integrity, we analyzed small-molecular-weight cadaverine leakage in Wntless cKO animals; we found no abnormalities in cortex or corpus callosum (Figures 5K and 5L). Similarly, we did not detect any fibrinogen deposits in brain parenchyma (Figure 5M) or alterations in the expression of the genes Plvap, Cltn5, Zic3, Foxf2, and Mfsd2a involved in BBB maturation and maintenance in WM tissue isolated from P8–P10 mice (Figure 5N) or major alterations in the EC junctional integrity, as indicated by PLVAP, Glut1, and Claudin 5 protein expression in WM vessels (Figure S6B). We observed a minor increase in GFAP* astrocyte reactivity but no significant changes in activated Iba1* microglial cell numbers or morphology (Figure S6C). These findings indicate that BBB integrity is maintained in Wntless cKO animals.

Figure 3. Increased Oligodendroglial Density Induces Hypervascularization of Developing White Matter in BrafF9A/ Genomic Mice

(A–C) DAPI-stained coronal sections from P0-CTRL and BrafF9A/ mutant mouse brains. BrafF9A/ mutants do not show any defects in the cortical or WM cytoarchitecture. High-magnification images of the cingulum region, highlighted in (B), shows increase in Olig2* oligodendroglial cells and CD31* BV density in BrafF9A/ mutants (C). Scale bars: 100 μm (B) and 50 μm (C).

(D and E) Quantification of WM Olig2* cells, and BV coverage, branching, and length in P0 mice.

(F) Forebrain regions in BrafF9A/ mice appear normal and do not show any neoplasms at P9. DAPI-stained coronal sections from CTRL and BrafF9A/ brains. Scale bar: 100 μm.

(F) High-magnification images from (F) showing sustained increase in Olig2* oligodendroglial cells and CD31* BV coverage in BrafF9A/ mutants. Scale bar: 50 μm.

(H and I) Quantification of WM Olig2* cells and BV coverage, branching, and length in P9 mice.

(J) PDGF-Rα+ OPCs form clusters on CD31* BVs in BrafF9A/ mutant WM; (bottom) isosurface rendering. Scale bar: 20 μm.

(K) Representative images of the WM region labeled with GFAP, aquaporin 4 (Aqp4), and collagen 4a (Col4a) in CTRL and BrafF9A/ mice. Note that no astrocyte endfeet coverage abnormalities were observed in BrafF9A/ mutants. Scale bar: 50 μm.

(L) Representative images of WM from P9-CTRL and BrafF9A/ brain sections labeled with Claudin 5 and Plasmalemma Vesicle Protein (PLVAP). Note the absence of Claudin 5* and PLVAP* vessels in WM, indicating no BBB damage. Inset (top) shows choroid plexus vessels from the same section as the PLVAP* expression control. Scale bar: 50 μm.

(M and N) Representative images of WM from P9 CTRL and BrafF9A/ mice injected with low molecular weight cadaverine-555 tracer (scale bar: 50 μm), and intensity quantifications showing no significant changes, i.e., no tracer leakage into brain parenchyma in BrafF9A/ mutants.

(O) Oligodendroglial cell density and vascular coverage in WM are positively correlated.

Data are represented as means ± SD and are analyzed with a two-tailed unpaired Student’s t test. n = 5 animals/genotype (D–I and O), n = 3 animals/genotype (N). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 4. Neonatal White Matter Injury-Induced Increase in Vascular Density Is Associated with Increased OPC Numbers and Upregulated Wnt7 Signaling

(A) Schematic for neonatal ferret chronic sub-lethal hypoxic injury paradigm.

(B) Hypomyelination in hypoxic ferret brain. Coronal brain sections from normoxic- (Nx) and hypoxic (Hx)-reared ferret kits stained with myelin basic protein (MBP). Scale bar: 500 μm.

(C and D) Increase in PDGFRα+Olig2+ OPCs (C) and decrease in immature BCAS1+Olig2+ OLs (D). Scale bars: 25 μm.

(E) Increased CD31+ BV coverage in the WM of Hx-ferrets. Scale bar: 25 μm.

(F) Quantification of oligodendroglial lineage cells and BV coverage, branching, and length in the WM tracts of Nx and Hx P20 ferrets.

(G) Cartoon showing the WM region in human hypoxic ischemic encephalopathy (HIE).

(H) Representative images of WM tracts of cingulate from human HIE and age-matched controls immunostained with BV marker collagen 4a (Col4a). Note a robust increase in vascular coverage in HIE. Scale bar: 50 μm.

(I) Quantification of WM vessel coverage and branching in CTRL and HIE cases.

(J–M) Increase in OPC (PDGFRα+Olig2+) (J and M) and decrease in immature and mature OL (BCAS1+Olig2+ or Nogo-A+Olig2+MBP+) (K–M) cell numbers in the WM tracts of human HIE cases. Scale bars: 25 μm.

(N) Cartoon showing hypoxia-induced cellular changes in the WM.

(O) Cartoon of OPC-induced endothelial Wnt signaling.

(P) Multiplex smFISH labeling of PDGFRα and Wnt7a in CTRL and HIE cases. Scale bar: 10 μm.

(Q) Multiplex smFISH of Wnt signaling downstream targets Apcdd1 and Axin2 and CD31 immunolabeling reveals an increase in EC Wnt signaling in WM tracts of HIE cases. Scale bar: 10 μm.

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through disrupted sprouting angiogenesis and EC proliferation (Figures 6I and 6J).

**OPC-Encoded Wntless Is Required for WM Integrity**

Because we found a decrease in Olig2+ cell numbers in the WM region of Wntless cKO animals, we analyzed whether this was due to oligodendroglial cell proliferation defects; however, we found no differences in Olig2+Ki67+ cell numbers at P12 (Figure S6D) or alterations in expression of canonical Wnt downstream targets Axin2 (Figure 7A) or TCF4 (Figure S6E). In contrast, we found a significant increase of apoptotic marker cl-caspase 3 in MBP+ OLs at P7 in both WM and deep cortical regions (Figure 7B). At P12, we found significantly decreased numbers of mature CC1+ and Plp1+ OL numbers (Figures 7C–7F), resulting in WM hypomyelination (Figure 7G). We also observed that Wntless cKO mice progressively displayed tremors, hind limb clamping, and reduced viability after the second postnatal week (Video S1). Analysis of WM at P18 (extent of survival) also revealed the appearance of axonal-damage marker SMI32 and APP+ axonal spheroids (Figures 7H and 7I). These findings suggest a model in which OPC-encoded Wntless function is essential for WM vascularity, OL survival, postnatal myelination, and ultimately, WM integrity.

**OPC-Encoded Wnt7a/b Function Attenuates WM Injury after Hypoxia**

We and others have reported roles for Wnt7a/b in regulating embryonic and postnatal CNS developmental angiogenesis (Cho et al., 2017, 2019; Daneman et al., 2009; Stenman et al., 2008; Yuen et al., 2014; Zhou and Nathans, 2014). To determine whether OPC-encoded Wnt7a/b function was essential during development or conferred resilience against hypoxic injury, we generated a compound-Wnt7a/b-deficient mutant mouse by intercrossing Olig2-Cre to conventional Wnt7a-null and conditional Wnt7b(Ifl/flf) alleles (hereafter Wnt7dKO) (Figure 8A), resulting in a significant decrease in Wnt7 ligand expression in oligodendroglial cells (Figure 8B).

Given that Wnt7dKO mice showed improved survival and only subtle developmental impact in WM versus Wntless cKO mice, we tested the effects of superimposed chronic neonatal hypoxemia (Hx) in Wnt7dKO mutant mice and controls (Figure 8C). As shown (Figures 8D and 8E), wild-type mice reared in hypoxic conditions (10% FiO2; Hx) showed the expected angiogenic response resulting in increased WM vascular density and branching. In dramatic contrast, that response and EC proliferation were lacking in hypoxic Wnt7dKO mice (Figures 8A, 8C, and 8B). We confirmed the downregulation of Wnt downstream targets Apcdd1 and Axin2 in these mutants (Figures S7A and S7B) and did not observe BBB disruption (absence of PLVAP protein expression changes and no cadaverine leakage) or gliosis in hypoxic Wnt7dKO mutants versus controls (Figures S7E–S7G). Of note, Wnt7a−/− conventional or Olig2-Cre/Wnt7b(Ifl/flf) conditional single mutants did not show a detectable phenotype after hypoxic insult (Figures S7H and S7I).

Because we observed a significant decrease in both oligodendroglial and vessel density in P11 hypoxic reared Wnt7dKO mutants (Figures 8D and 8F), we investigated the timing of that decline. At P7, hypoxic reared Wnt7dKO mice began to show vascular density deterioration, whereas oligodendroglial cell density remained unchanged (Figures S8A and S8B). Similar to results above with Wntless cKO animals, we observed increased apoptosis in WM of hypoxic Wnt7dKO mutants as well as reduction in CC1+ and Plp1+ mature OLs, severe hypomyelination, increased expression of axonal damage marker SMI32, and reduced WM volume (Figures 8G–8M and S8C). In contrast to WM, cortical oligodendroglial and OPC numbers remained unchanged (Figures S8D and S8E). Together, these findings indicate that OPC-encoded Wnt7 function is a critical determinant of WM susceptibility to hypoxic injury. They do not exclude roles for other OPC-encoded Wnt ligands or other OPC-derived angiogenic factors in the regulation of developmental WM angiogenesis in normoxic or hypoxic conditions.

**DISCUSSION**

Here, we addressed the fundamental question of how vascular density is orchestrated in mammalian cortical WM and implications of this for resilience against hypoxic insult in neonatal brain. A major insight is that OPC-endothelial-tip-cell interactions have a direct role in this process and that alterations in OPC density and angiogenic signaling drive vascular remodeling in the WM. We show that oligodendroglial-EC interactions attenuate susceptibility to hypoxic neonatal brain injury in a Wnt-dependent manner, a finding with implications for human neonatal WM injury, cerebral palsy, and WM stroke. These and other findings discussed below indicate bi-directional crosstalk between OPCs and vasculature during development and in disease.

**Evidence for OPC-Endothelial Bi-directional Crosstalk in WM**

Vascular investment of the primordial brain regions depends on neuroepithelial and radial glial angiogenic cues, including canonical Wnt signaling (Gruzdzendor et al., 2014; Lam et al., 2010; Ma et al., 2013; Stenman et al., 2008). The embryonic vasculature is subsequently remodeled at neonatal stages by neuronal and glial populations (Bozoyan et al., 2012; Harb et al., 2013; Ma et al., 2013; Paredes et al., 2018; Segarra et al., 2018; Vasudevan et al., 2008), including NG2 glia (Minocha et al., 2015). Several studies show that OPC-encoded HIF function regulates cell-autonomous OPC maturation and myelination as well as WM angiogenesis via paracrine signaling (Allan et al., 2020; Yuen et al., 2014; Zhang et al., 2020). Here, we extend insights into this process and show the OPCs and BVs engage in further cell-cell interactions in the neonatal brain. Strikingly, we found that OPCs form what appear by ultrastructure to be direct and

(R) Quantification of Apcdd1 and Axin2 RNA spots in CD31+ EC nuclei.

Data are represented as means ± SD and are analyzed with a two-tailed unpaired Student’s t test. n = 3 animals/condition in (F), n = 6 cases/condition in (I) and (M), and n = 3 cases/condition for (R). Violin plots in (R) represent RNA spots quantified from at least 100 cells from three different control and HIE cases, and overlaying data points represent the average number of RNA spots from each case. *p < 0.05, **p < 0.01.

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Figure 5. OPC Wnt Activity Is Necessary for Postnatal White Matter Vascular Development.

(A) Wntless in the endoplasmic reticulum promotes Wnt ligand transport to the plasma membrane where they are secreted.

(B) Schematic of Wntless conditional knockout (Wntless cKO) generation.

(C and D) White matter development in Wntless cKO is not affected at P3, as shown by Olig2+ and CD31+ labeling and quantification. Scale bar: 50 μm.

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frequent contacts with sprouting endothelial tip cells. Our data suggest tight coupling of OPC number and WM angiogenesis in transgenic mouse lines with decreased or increased OPC numbers. Together, these findings firmly establish OPCs as major regulators of WM angiogenesis.

Embryonic OPCs depend on the vasculature for distribution throughout the CNS (Tsai et al., 2016), indicating that bi-directional crosstalk is essential during development. Moreover, during remyelination clustering of OPCs along BVs in multiple sclerosis (MS) lesions has been observed (Niu et al., 2019), and a recent study elucidated the role for ECs in adaptive myelination (Swire et al., 2019). Other lines of evidence indicate this relationship persists postnatally until adult stages. For example, vascular dysfunction underlies WM abnormalities in various pathological settings (Montagne et al., 2018; Rajani and Williams, 2017). Further research is warranted to investigate the complex inter-relationships between the oligodendroglial lineage and the vasculature during development and in neurological diseases.

**OPC-Encoded Wnt Signaling Is Essential for Neonatal WM Angiogenesis and Axonal, but not BBB, Integrity**

Angiogenesis in the mouse brain is robust from E9.5 until the second postnatal week (Paredes et al., 2018). Although the role of embryonic Wnt signaling and radial glia in regulation of CNS vasculature is known (Cho et al., 2017, 2019; Daneman et al., 2009; Stenman et al., 2008; Zhou and Nathans, 2014), postnatal angiogenic roles for Wnt signaling in OPCs are incompletely understood. Here, we incapacitated all Wnt ligand production by conditionally targeting Wntless with Olig1-cre, which drives activity in OPCs (Silbereis et al., 2014). Interestingly, although OPC number was unaffected in the WM of P7 Wntless mutants, we found a severe effect on tip cell development and BV density, indicating that OPC-encoded Wnt activity is essential for neonatal angiogenesis. In contrast to normoxic Olig1-cre/Wntless mice, Olig2-cre, Wnt7dKO had a mild phenotype without effect on the developing vasculature. Because Wntless is required for secretion of all Wnt ligands and given that oligodendroglia also express Wnt3, 4, 6, 9a, and 10a (Zhang et al., 2014), other Wnts besides Wnt7 might compensate for the loss of Wnt7a/b during normal neonatal WM angiogenesis.

In contrast, specific requirements for Wnt7a/b were indicated in the setting of hypoxic injury. We found that chronic hypoxia in mouse and ferret as well as human HIE cases showed significantly increased OPC and vessel density in WM tracts, coupled with an increase in canonical Wnt/β-catenin targets Lef1, Apcdd1, and Axin2 in ECs. In human HIE lesions, we observed WNT7A-expressing OPCs. Additionally, Wnt7a/b mutants in the hypoxic setting showed dramatic abrogation of vessel induction and severe damage to WM and hypomyelination, suggesting specific roles for OPC-encoded Wnt7 function to maintain resilience against hypoxic injury.

Canonical Wnt signaling in CNS ECs is required for BBB maturation in a region-restricted manner (Cho et al., 2017; Wang et al., 2012, 2018; Zhou and Nathans, 2014; Zhou et al., 2014); for instance, loss-of-Frizzled 4 function results in BBB disruptions only in the cerebellum, retina, and olfactory bulb (Wang et al., 2012). We found that neither alterations in OPC density or deletion of OPC-encoded Wnt ligand activity resulted in BBB disruption. This indicates that OPC-Wnt activity acts primarily to support endothelial tip cell development and vessel growth, but not BBB integrity.

**OPC-Wnt Is a Primary Angiogenic Signal That Acts in a Paracrine Manner in Neonatal WM**

Wnt ligands act as short-range signals (Clevers et al., 2014) and have specific roles in brain angiogenesis (Vanhollebeke et al., 2015). We found that the loss-of-OPC Wnt function in both Wntless and Wnt7dKO animals resulted in decreased WM vessel density and downregulation of canonical Wnt/β-catenin targets Apcdd1 and Axin2 in ECs, indicating cell-to-cell signaling. Moreover, OPC-Wnt signaling was required specifically to maintain expression of Apelin in angiogenic tip cells in contrast to other pro-angiogenic cues in Wntless mutants. A recent study has shown Apelin signaling promotes a pro-angiogenic state in zebrafish endothelial cells (Helker et al., 2020), and interestingly, loss of Apelin function caused tip cell defects similar to those we observed in OPC-Wntless mutants. While autocrine Wnt signaling inhibits OPC differentiation (Fancy et al., 2009, 2011, 2014; Hammond et al., 2015), we found that expression of the Wnt downstream target Axin2 was unaltered within the OPCs themselves in Wnt mutant animals, and we did not observe precocious OPC differentiation; thus, we found no evidence for autocrine effects. Because OPCs form intimate contacts with endothelial tip cells, these findings collectively indicate OPC-Wnt acts in a paracrine manner to regulate WM vascular development.

**Therapeutic Implications**

OPCs are involved in a wide spectrum of injuries, including neonatal HIE (Billiards et al., 2008; Fancy et al., 2011), stroke...
(Chen et al., 2018; Joseph et al., 2016), and MS (Fancy et al., 2011), pathologies associated with chronic hypomyelination. As angiogenesis is a fundamental initial response to a variety of tissue insults, the OPC-vascular crosstalk we describe here might also be adaptive, because OPCs can rapidly migrate along BVs into brain lesions (Niu et al., 2019). Indeed, our data suggest a new role for OPCs in this context, namely, directly regulating angiogenesis in the setting of WM lesion repair. Because several studies have shown abnormal OPC Wnt signaling in human neonatal WM injury and MS (Fancy et al., 2009, 2014), an implication of this study is that post-injury angiogenic response could be compromised.

WM ischemic stroke is a leading cause of motor dysfunction and cognitive impairment (Román et al., 2002), and studies in experimental models have shown that OPC numbers are upregulated during the recovery phase of the injury (Joseph et al., 2016; Sozmen et al., 2016; Zhang et al., 2011); indeed, OPC neuroprotection during the acute phase of stroke injury improves functional recovery (Chen et al., 2018). Interestingly, OPCs have been suggested to have a beneficial role in recovery by promoting post-injury angiogenesis and BBB rescue in various stroke injury models (Kishida et al., 2019; Wang et al., 2020).

As to the nature of secreted pro-angiogenic cues, the current study indicates a specific function for OPC-encoded Wnt7.

Members of the VEGF family are well-established hypoxic signaling targets (Himmels et al., 2017) that show developmental expression within OPCs (Cahoy et al., 2008). Indeed, VEGF expression has also been reported to be regulated by canonical
Figure 7. Disruption in Oligodendroglial Intrinsic Wnt ligand Activity Results in Loss of Mature Oligodendrocytes and Hypomyelination.

(A) Multiplex smFISH of Plp1, PDGFRa, and Axin2 shows no alterations in oligodendroglial intrinsic canonical Wnt/β-catenin activity in Wntless cKO mice. PDGFRa+ OPCs are outlined in white. Scale bar: 5 μm.

(B) Cortical WM regions labeled with MBP and cleaved caspase 3 show increase in OL apoptosis in the WM and deep cortex regions in Wntless cKO mice at P7. Insets show highlighted regions. Scale bars: 100 μm and 50 μm for insets. Right panel shows quantification of cleaved caspase 3+ cells in WM.

(C and D) Significant loss of mature OLs (CC1+Olig2+ cells) in Wntless cKO animals at P12. Scale bar: 50 μm.

(E and F) Significant reduction in mature OLs expressing Plp1 mRNA in Wntless cKO at P12. Scale bar: 50 μm.

(G) Hypomyelination of Wntless cKO animals at P12 revealed by myelin basic protein (MBP) and pan-neurofilament marker (NF) immunostaining. Scale bar: 50 μm.

(H) Increase in expression of axonal damage marker SMI32 in the corpus callosum of Wntless cKO mice at P18. Scale bar: 10 μm.

(I) Appearance of amyloid precursor protein (APP+) axonal spheroids in P18 Wntless cKO mice (indicated by black arrowheads), indicating axon damage; corpus callosum and external capsule regions are shown. Scale bar: 10 μm.

Data are represented as means ± SD and are analyzed with a two-tailed unpaired Student’s t test. n = 6 animals/genotype for data represented in (B) and n = 5 and 4 each for data represented in (D) and (F), respectively. **p < 0.01, ***p < 0.001.
Wnt signaling (Easwaran et al., 2003; Wu et al., 2015). Similarly, other pro-angiogenic candidate factors, such as semaphorins and neuropilins, are also expressed by oligodendroglial lineage cells. Because, aged patients show diminished recovery from stroke compared with young adults (Bousser, 2012; Ovbiagele and Nguyen-Huyhn, 2011) and OPC functions decline in aged animals (Ruckh et al., 2012), a related question is how OPC angiogenic factor production is regulated in aging? Future work is needed to investigate OPC-encoded regulators of BV development over the life course and their functional relevance in neonatal, juvenile, and/or adult WM injuries.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2020.09.033.

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AUTHOR CONTRIBUTIONS

M.C. and D.H.R. conceived the original idea. M.C. designed and performed all experiments and data analysis. M.J.U.-N. and J.M.G.-V. performed Olig2 immunogold labeling and electron microscopy studies. P.P.-B. performed surgical resection of the white matter tissue used for electron microscopy studies. P.S.M. and E.J.H. provided advice on experimental design related to ferret hypoxic injury and human HIE experiments, respectively. M.C. and D.H.R. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-Olig2    | Dr. Charles D. Stiles (Arnett et al., 2004) | RRID: AB_10807410 |
| Goat anti-Olig2     | R&D Systems | Cat# AF2418; RRID: AB_2157554 |
| Rabbit anti-CD31/PECAM | ABCAM | Cat# ab28365; RRID: AB_726365 |
| Rat anti-CD31/PECAM | BD Biosciences | Cat# 550274; RRID: AB_393571 |
| Mouse anti-CD31/PECAM | ABCAM | Cat# ab187377; RRID: AB_2756834 |
| Rabbit anti-ERG     | Cell Signaling | Cat# 97249; RRID: AB_2721841 |
| Rabbit anti-PDGFrA  | Cell Signaling | Cat# 3164; RRID: AB_2162351 |
| Goat anti-Collagen 4a | Millipore | Cat# ab769; RRID: AB_92262 |
| Rabbit anti-Iba1    | Wako | Cat# 019-19741; RRID: AB_839504 |
| Mouse anti-Claudin 5 | Thermo Fisher Scientific | Cat# 35-2500; RRID: AB_2533200 |
| Chicken anti-GFP    | Aves Labs | GFP-1020; RRID: AB_10000240 |
| Rabbit anti-Aquaporin-4 | Sigma Aldrich | Cat# A5971; RRID: AB_258270 |
| Rabbit anti-Glut1   | Millipore | Cat# 07-1401; RRID: AB_1587074 |
| Rat anti-PLVAP (MECA-32) | BD Biosciences | Cat# ab118533; RRID: AB_10900171 |
| Sheep anti-Fibrinogen | ABCAM | Cat# ab118533; RRID: AB_10900171 |
| Mouse anti-APC (CC-1) | Millipore | Cat# OP80; RRID: AB_2057371 |
| Rat anti-GFAP (2.2B10) | Invitrogen | Cat# 13-0300; RRID: AB_2532994 |
| Mouse anti-BCAS1    | Santa Cruz | Cat# sc-136342; RRID: AB_10839529 |
| Mouse anti-Nogo-A   | R&D Systems | MAB3098; RRID: AB_10997139 |
| Rat anti-MBP        | Millipore | Cat# MAB386; RRID: AB_94975 |
| Rabbit anti-Cleaved Caspase 3 | Cell Signaling | Cat# 9661; RRID: AB_2341188 |
| Goat anti-Wnt7      | Santa Cruz | Cat# sc-26361; RRID: AB_2215743 |
| Mouse anti-CD68     | BD Biosciences | Cat# 556059; RRID: AB_396329 |
| Mouse anti-Ki67     | BD Biosciences | Cat# 550609; RRID: AB_393778 |
| Rabbit anti-Lef1    | Cell Signaling | Cat# 2230; RRID: AB_823558 |
| Mouse anti-SMI32 (Nonphosphorylated NF-H) | Biolegend | Cat# 801702; RRID: AB_2715852 |
| Chicken anti-Neurofilament H | Encor Biosciences | Cat# CPA1-NF-H; RRID: AB_2149761 |
| Mouse anti-APP (A4) | Millipore | MAB348; RRID: AB_94882 |
| Mouse anti-TCF4     | Millipore | Cat# 05-511; RRID: AB_309772 |
| Donkey Anti-mouse IgG (H+L), Alexa Fluor 488, 555, 647 | Thermo Fisher Scientific | Cat# A-21202; RRID: AB_141607, # A-31570, RRID: AB_2536180, # A-31571, RRID: AB_162542 |
| Donkey Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 488, 555, 647 | Thermo Fisher Scientific | Cat# A-21206; RRID: AB_2535792; A-31572, RRID: AB_253643; A-31573, RRID: AB_2536183 |
| Donkey Anti-Goat IgG (H+L), Alexa Fluor 488, 594, 647 | Thermo Fisher Scientific | Cat# A-11055, RRID: AB_2534102, A-21432, RRID: AB_2535853, A-21447, RRID: AB_2535864 |
| Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11042; RRID: AB_2534099 |
| Goat anti-rabbit IgG (H+L) secondary antibody, Alexa fluor 488,555,647 | Thermo Fisher Scientific | Cat# A-11008; RRID: AB_143165; A-21428, RRID: AB_2535849; A-21244, RRID: AB_2535812 |
| Rabbit anti-Olig2   | Millipore | Cat# AB9610; RRID: AB_570666 |
| Goat anti-Rabbit IgG Gold (H&L) Ultra Small | Aurion | SKU: 800.011 |

(Continued on next page)
**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David H Rowitch (dhr25@medschl.cam.ac.uk).

**Materials Availability**
All unique reagents/materials generated in this study will be available from the Lead contact upon completion of pertinent material transfer agreement.
**Data and Code Availability**
This study did not generate any code.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

All animal procedures were performed according to the University of California at San Francisco guidelines under Institutional Animal Care and Use Program (IACUC)-approved protocols. Age of the mice/ferrets used for the study are identified in the main text and figure legends. Animals were housed under standard 12-hour light/dark cycle conditions and were fed ad libitum. The following mouse lines were used in the study: 

- **Olig2-cre** mouse line was previously described (Schüller et al., 2008). Briefly, the line was generated by inserting an avian-specific retroviral receptor, and an IRES-cre recombinase cassette into the endogenous Olig2 locus by homologous recombination. Sox10-DTA mice have been generated by Dr. William Richardson’s lab and are previously described (Kessaris et al., 2006). 

- BRAFCA is a knock-in allele of human BRaf which expresses normal BRaf prior to Cre recombinase exposure and encodes mutationally activated BRAFV600E after Cre recombination (Dankort et al., 2009). 

- **Olig1-Cre** mouse line was generated by inserting a cre-neo cassette into the Olig1 locus via homologous recombination and was previously described (Lu et al., 2002). 

- Sox10-DTA mice were purchased from Jackson laboratory and are previously described (Carpenter et al., 2010). 

- **Wnt7aKO** mice were generated by intercrossing Olig2-Cre mouse line to Wnt7a+/− and Wnt7b (fl/fl) lines (Stenman et al., 2008). Note that Wnt7a−/− mice are infertile so they were bred as heterozygotes. For experiments involving transgenic animals, littermates were randomly assigned to experimental groups with approximately equal amounts of male and female animals.

Chronically hypoxic rearing was performed as previously described (Yuen et al., 2014). Briefly, litters from Control or Wnt7aKO animals were culled to a size of 4 pups and co-fostered with lactating CD1 strain dams then reared at 10% O₂ in a hypoxic chamber starting on postnatal day 3 (P3) (Biospherix, Inc., Laconia, NY). Dams were provided ad libitum access to water and food. Mice were monitored daily to ensure that there are no signs of distress, poor intake of food and water, unusual/decreased grooming. Tissue from P11 pups were then harvested for analysis with in 1 hour after removal from hypoxia chamber.

To perform ferret chronic sublethal hypoxia experiments, pregnant jills were obtained from Marshall Farms (North Rose, NY) at E26 gestation. The kits from these ferrets along with the jill were placed in a 10% oxygen chamber on postnatal day 10 (P10) until P20 endpoint. The health of kits and jill were monitored daily (lethargy, poor weight gain and failure to nurse). Animals were provided ad libitum access to water and food. Mice were monitored daily to ensure that there are no signs of distress, poor intake of food and water, unusual/decreased grooming. Tissue from P11 pups were then harvested for analysis with in 1 hour after removal from hypoxia chamber.

**Human Neonatal Brain Specimen**

This study involves use of de-identified human post-mortem fixed-frozen brain tissue samples obtained from University of California, San Francisco’s Pediatric Neuropathology Research Laboratory (see Table S1 for clinicopathological information). Tissue was fixed with 4% paraformaldehyde, followed by sequential immersion in 10%, 20% and 30% sucrose for cryopreservation. Frozen tissues are sectioned at 14 μm for immunohistochemistry and smFISH applications. All the HIE cases in this study showed evidence of diffuse white matter injury, with astrogliosis and macrophage infiltration (Figure S5). It must be noted that although some control brain specimens were obtained from diaphragmatic hernia cases, which may result in hypoxemia, these specimens were examined and classified as “control” by an experienced neuropathologist and did not exhibit any evidence of astrogliosis or macrophage infiltration. Both male and female cases from control and HIE conditions were analyzed, and we did not observe any gender association on the results reported.

**METHOD DETAILS**

**Tissue Processing and Immunohistochemistry**

Mice were deeply anesthetized and transcardially perfused with cold PBS followed by cold 4% PFA. Brains were isolated and post-fixed overnight in 4% PFA and cryo-protected in 30% sucrose for 24 hours and then frozen at −80 °C until sectioned. Frozen brains were sectioned at 30 μm thickness. Tissue sections were blocked for 1 hour using blocking solution (5% horse serum/0.3% Triton X-100 in PBS) and incubated with primary antibodies (resource table) overnight at 4 °C in fresh blocking solution. Sections were washed thrice in PBS containing 0.4% Triton X-100 followed by incubation with the appropriate secondary antibodies purchased from Invitrogen. Three to five additional washes were performed, and sections were mounted using DAPI-Fluoromount G (Southern-Biotech). Antigen retrieval was performed by pre-treating the sections for 10 minutes at 95 °C in 10mM Sodium Citrate (pH 6.0) in a microwave oven (BioWave). Histological preparations were analyzed using Leica Sp5 upright AOBS confocal microscope. Images were acquired with either 20x, 40x or 63x objectives.

**Olig2 Immunogold Labeling and Electron Microscopy Processing**

The specimen was obtained by a surgical resection of the right temporal lobe from a 2-year old female with type IIB focal cortical dysplasia. The tissue was fixed in 4% PFA in 0.1M PBS for 7 days. After 100 μm sections were obtained using a using a Leica
VT1000S vibratome (Leica Biosystems, Wetzlar, Germany). For pre-embedding immunogold, the tissue was cryoprotected in a solution containing 25% saccharose in 0.1M phosphate buffer (PB) for 30 min followed by repeated freeze-thaw cycles for permeabilization. The permeabilization step was performed by immersing the samples repeatedly in −20°C 2-methylbutanol and transferring them to room temperature saccharose solution. After, the samples were incubated in a blocking solution consisting of 0.3% BSaC (Aurion, Wageningen, the Netherlands), 0.05% sodium azide in 0.1 M PB for 1 h. Subsequently, the samples were incubated in primary antibody (1:150 rabbit anti-Olig2, Millipore) in blocking solution for 72 h at 4°C. The sections were then rinsed in 0.1 M PB and incubated in secondary antibody blocking solution consisting of 0.5% BSaC (Aurion), 0.025% CWSF gelatin (Aurion), 0.05% sodium azide in 0.1 M PB for 1 h, followed by incubation in secondary antibody (1:50 goat-anti-rabbit IgG gold ultrasmall; Aurion) diluted in the same solution overnight at 4°C. To enhance gold labeling, we performed silver enhancement (R-GENT SE-LM, Aurion) for 15–25 min in the dark, followed by gentle washing in 2% sodium acetate and incubation in gold toning solution (0.05% gold chloride in water) for 10 min. The sections were then washed twice with 0.3% sodium thiosulfate in water. Finally, we post fixed with 2% glutaraldehyde (Electron Microscopy Sciences) in 0.1M PB for 30 min.

For transmission electron microscopy analysis, specimen was post fixed with 1% osmium tetroxide (Electron Microscopy Sciences), 7% glucose in 0.1M PB for 30 min at room temperature, washed in deionized water, and partially dehydrated in 70% ethanol. Afterward, the samples were contrasted in 2% uranyl acetate (Electron Microscopy Sciences) in 70% ethanol for 2 hours at 4°C. The samples were further dehydrated and embedded in Durcupan ACM epoxy resin (Sigma-Aldrich) at room temperature overnight, and then at 60°C for 72 h. Once the resin was polymerized, immunolabeled sections were selected and cut into semithin (1.5 μm) and ultrathin (60–80 nm) sections using a UC6 ultramicrotome (Leica Biosystems). These sections were placed on Formvar-coated single-slot copper grids (Electron Microscopy Sciences) stained with lead citrate and examined at 80 kV on a FEI Tecnai G2 Spirit (FEI Company, Hillsboro, OR) transmission electron microscope equipped with a Morada CCD digital camera (Olympus, Tokyo, Japan).

Single molecule fluorescent in situ hybridization
Three-color smFISH was performed on fixed frozen sections from human neonatal brain specimen or control and mutant mice using Advanced Cell Diagnostics RNAscope® Fluorescent Multiplex Reagent Kit and probes. Cryosections (14 μm thick) were mounted on glass slides and washed in RNase free PBS for 5 mins. Slides were then baked at 60°C for 30 mins and were further fixed in 4% neutral buffered paraformaldehyde for 15 min at 4°C. Next, sections were dehydrated in 50%, 70% and 100% ethanol for 5 mins at room temperature and air-dried. Target retrieval was then performed with RNAscope reagents for 5 mins at 95°C for human and 2 minutes for mouse tissue respectively and sections were further washed with distilled water followed by washes in 100% ethanol 2-3 times. Sections were next treated with Protease IV reagent for 30 mins at 40°C (for human tissue) or Protease III reagent for 15 minutes at 40°C (for mouse tissue). Sections were then washed and maintained in RNase free water until hybridization step. Probes listed in the resource table were diluted at 1:50 ratio in channel 1 probe and preheated to 40°C for 5 mins and sections were incubated with this probe mix for 2hr at 40°C. After probe hybridization, sections were washed twice for 2 mins each before proceeding to the fluorescent detection step according to manufactures protocol. Briefly sections were incubated in AMP 1-FL for 30 min at 40°C, washed two times, incubated in RNAscope AMP 2-FL for 15 min at 40°C, washed two times, incubated in RNAscope AMP 3-FL for 30 min at 40°C, washed two times and incubated in AMP 4-FL-Alt B solution for 15 mins at 40°C, washed two times before IHC labeling (see above). If IHC was not required, sections were counterstained with RNAscope DAPI for 30 s after final detection step. All wash steps were performed with RNAscope 1x wash buffer. Quantification of RNA spots was performed on images acquired at 63x on Leica SP5 upright AOBs confocal microscope. RNAspots were quantified from atleast 100 cells from images obtained from different human cases/condition or animals/genotype and represented as SuperPlots (Lord et al., 2020).

Cadaverine Permeability Assay
Lysine-fixable, cadaverine conjugated to Alexa Fluor-555 (ThermoFischer Scientific) was injected intraperitoneally at 25mg/kg and the mouse pups were sacrificed after 2 hours and the brains were isolated as described above. Cadaverine leakage was quantified by mean pixel intensity, measured from different microscopic fields from atleast 3 sections from three animals each per genotype.

Quantitative PCR Analysis
Forebrain white matter tissue isolates were obtained from P3, P8-P10 control or Wntless cKO mice and lysed with Qiazol (QIAGEN) and RNA was isolated with the RNeasy Kit (QIAGEN) following manufacture’s protocol. Total RNA was reverse transcribed to cDNA using SuperScript IV First-Strand Synthesis System (Invitrogen) for quantitative-PCR experiments (qPCR). Using the transcript specific primers (see Table S2), cDNA was amplified, and qPCR was performed using the Roche 480 LightCycler and SYBR Green-based Master Mix. Gene expression levels were calculated using the ΔΔCt method and normalized using GAPDH as a reference.

Vascular Density, Branching and Length Quantifications
Images were obtained as above from regions of interest (Corpus collosum, cingulum and external capsule from various rostro-caudal levels) and vascular coverage was analyzed on ImageJ. Vessel images were segmented on ImageJ and the percentage of vessel coverage was determined by quantifying the percent of the vessel segmented area over the total imaged area. Vessel branching...
was manually counted from the images obtained from at least 40-50 fields from different white matter regions from n = 4-7 animals as mentioned in figure legends and presented as average number of branches per unit area quantified. Total vascular length was quantified as the sum of lengths of all vessels in a field.

Microscopy and Cell Counting
Optical sections of confocal epifluorescence images were sequentially acquired using LAS AF software (Leica). Images were merged using ImageJ software and merged images were processed on Adobe Illustrator software. Background modifications, where required for presentation purposes, were applied evenly across the entire image and between the control and experimental groups using Leica’s LAS software or ImageJ. At least n = 4-7 different animals for each experimental condition were collected and analyzed as indicated in the respective figure legends. Matched sections between −2.5 and +1.10 bregma were used for analysis and the regions indicated in figures were analyzed. Typically, 3-6 fields from 6-8 non-adjacent sections were analyzed per animal and counts are presented as the number of cells per 1 mm² or percentage of cells within the indicated cell population. All counts were performed using ImageJ software. All cell counts were performed with the researcher blinded to condition.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data are represented as mean ± SD and the number of experiments, cases analyzed and animals per genotype and condition are indicated in all figure legends. No statistical methods were used to predetermine the sample size. No data were excluded from analyses. Statistical analysis was performed using the two-tailed unpaired Student’s t test. A “p” value < 0.05 was considered significant. In all cases, * indicates a p value < 0.05, ** < 0.01, and ***p < 0.001, **** p < 0.0001. All the data analysis and plotting were performed on GraphPad Prism version 6.0.