

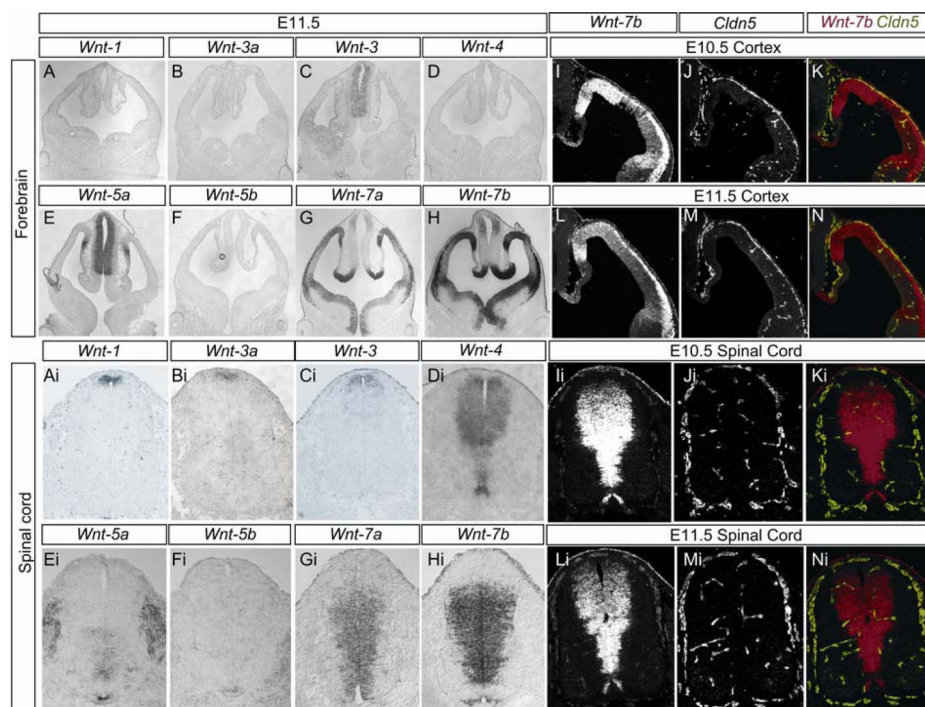
## Corrections

## NEUROSCIENCE

Correction for “Wnt/ $\beta$ -catenin signaling is required for CNS, but not non-CNS, angiogenesis,” by Richard Daneman, Dritan Agalliu, Lu Zhou, Frank Kuhnert, Calvin J. Kuo, and Ben A. Barres, which appeared in issue 2, January 13, 2009, of *Proc Natl Acad Sci USA* (106:641–646; first published January 7, 2009;

10.1073/pnas.0805165106).

The authors note that in Fig. 2Bi an image of Wnt1 in situ hybridization was mistakenly inserted instead of an image of Wnt3a in situ hybridization. This error did not affect the conclusions of this article. The corrected figure appears below.



**Fig. 2.** Expression of Wnts in the developing mouse CNS. In situ hybridizations demonstrating Wnt ligand expression in the developing forebrain (A–H) and spinal cord (Ai–Hi) of E11.5 mice. Canonical Wnt ligands Wnt7a and Wnt7b are expressed by neural progenitors in the ventricular zone in the ventral-lateral spinal cord and cortical forebrain, whereas canonical Wnt ligands Wnt1, Wnt3, and Wnt3a are expressed by neural progenitors in the ventricular zone of the dorsal spinal cord and the hindbrain. Non-canonical Wnt ligands Wnt4, Wnt5a, and Wnt5b are also expressed by neural progenitors located in spatially distinct regions of the spinal cord and cortex. Double fluorescent in situ hybridizations in the developing forebrain (E10.5 I–K, E11.5 L–N) and spinal cord (E10.5 Ii–Ki, E11.5 Li–Ni) with Wnt7b (I, L, Ii, Li) and Claudin 5 (J, M, Ji, Mi) and merged (K, N, Ki, Ni) demonstrate that claudin 5 positive vessels vascularize Wnt7b positive regions of the developing CNS.

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# Wnt/ $\beta$ -catenin signaling is required for CNS, but not non-CNS, angiogenesis

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Despite the importance of CNS blood vessels, the molecular mechanisms that regulate CNS angiogenesis and blood–brain barrier (BBB) formation are largely unknown. Here we analyze the role of Wnt/ $\beta$ -catenin signaling in regulating the formation of CNS blood vessels. First, through the analysis of TOP-Gal Wnt reporter mice, we identify that canonical Wnt/ $\beta$ -catenin signaling is specifically activated in CNS, but not non-CNS, blood vessels during development. This activation correlates with the expression of different Wnt ligands by neural progenitor cells in distinct locations throughout the CNS, including Wnt7a and Wnt7b in ventral regions and Wnt1, Wnt3, Wnt3a, and Wnt4 in dorsal regions. Blockade of Wnt/ $\beta$ -catenin signaling *in vivo* specifically disrupts CNS, but not non-CNS, angiogenesis. These defects include reduction in vessel number, loss of capillary beds, and the formation of hemorrhagic vascular malformations that remain adherent to the meninges. Furthermore, we demonstrate that Wnt/ $\beta$ -catenin signaling regulates the expression of the BBB-specific glucose transporter glut-1. Taken together these experiments reveal an essential role for Wnt/ $\beta$ -catenin signaling in driving CNS-specific angiogenesis and provide molecular evidence that angiogenesis and BBB formation are in part linked.

blood vessel | blood-brain barrier | Wnt | glut-1 | transport

Angiogenesis, the process by which new blood vessels are generated from existing blood vessels, is critical to ensure the supply of oxygen and nutrients to many tissues throughout the body. This process is especially important for the CNS as the neural tissue is extremely sensitive to hypoxia and ischemia. The blood vessels in the brain form a specialized structure, termed the blood brain barrier (BBB), which limits the flow of molecules and ions from the blood to the brain (1, 2). This BBB is critical to maintain brain homeostasis and protect the CNS from toxins and pathogens. CNS endothelial cells which form the BBB differ from endothelial cells in non-neural tissue, in that they are highly polarized cells held together by tight junctions that limit the paracellular flow of molecules and ions (1, 2). In addition, CNS endothelial cells also express specific transporters, both to provide selective transport of essential nutrients across the BBB into the brain and to efflux potential toxins from the brain (1, 2). Transplantation studies have demonstrated that the properties of the BBB are not intrinsic to the endothelial cells but induced by signals from the CNS parenchyma (3, 4). This has led to a two step model for BBB induction, described as angiogenesis followed by barrierogenesis (2). In this model, angiogenesis in the CNS proceeds by the same mechanisms as angiogenesis in non-neural tissues with the formation of leaky vessels, which are then induced to form the barrier by interactions with neural cells. Recent evidence, however, suggests that BBB formation occurs early during embryogenesis (5), raising the possibility that distinct molecular mechanisms may regulate CNS angiogenesis to tightly couple this process to BBB formation.

We have identified that several effectors of Wnt signaling are highly enriched in brain endothelial cells compared with the peripheral endothelial cells of the liver and lung, suggesting that Wnts may specifically regulate CNS vessel formation and/or function. Wnts are a highly conserved gene family that encode lipid modified secreted proteins; there are 19 known members of this

gene family in mice (7). During canonical Wnt signaling, binding of Wnt ligands to Frizzled/LRP receptor complexes causes a stabilization of  $\beta$ -catenin, which is normally degraded by Axin/GSK-3/APC complexes. Stabilized  $\beta$ -catenin is then able to translocate to the nucleus and through interactions with the TCF/LEF-1 complexes, regulates the expression of specific genes (7). These genes are involved in cell proliferation, differentiation, adhesion, morphogenesis, and other processes that are involved in the development, homeostasis, and disease of many tissues and organs.

Several studies have implicated Wnt/ $\beta$ -catenin signaling in regulating angiogenesis. In cell culture, endothelial cells have been shown to express Wnt ligands, receptors, and secreted modulators, and Wnt5a has been shown to regulate endothelial cell survival, proliferation, and gene expression (8–10). These studies have used endothelial cells immortalized from several different tissues which implies that Wnt signaling may regulate general endothelial cell function. Our genomic data, however, suggests that canonical Wnt/ $\beta$ -catenin signaling may specifically act on CNS vessels. Interestingly, Wnts have also been implicated in regulating angiogenesis in the placenta and gonads *in vivo* (11, 12). Both the placenta and gonads are tissues where blood tissue barriers are critical to their physiological function.

Here we analyze the function of Wnt signaling in regulating the formation of cerebral vessels *in vivo*. First, using TOP-GAL Wnt reporter mice, we show that canonical Wnt signaling is indeed activated in CNS endothelial cells, but not in non-neural endothelial cells, during development. We demonstrate that Wnt is a potent migration signal for CNS endothelial cells, and that inhibition of Wnt/ $\beta$ -catenin signaling *in vivo* leads to severe CNS specific angiogenesis defects without affecting non-CNS angiogenesis. Lastly, we demonstrate that Wnt/ $\beta$ -catenin signaling regulates the expression of the BBB-specific transporter glut-1. Taken together, these data suggest an important role for Wnt signaling in the formation of specialized CNS blood vessels and provide a molecular link between CNS angiogenesis and induction of BBB properties.

## Results

**Wnt Signaling Is Activated in CNS Vessels During Embryogenesis.** To confirm that Wnt signaling is indeed activated specifically in CNS vessels, we analyzed tissue sections from mice expressing the Wnt reporter TOP-GAL transgene. These transgenic mice express the lacZ gene under the control of Tcf promoters and thus synthesize lacZ only in cells in which canonical Wnt/ $\beta$ -catenin signaling is activated (13). During the development of the murine CNS, angiogenesis is initiated at E10, as endothelial cells from the

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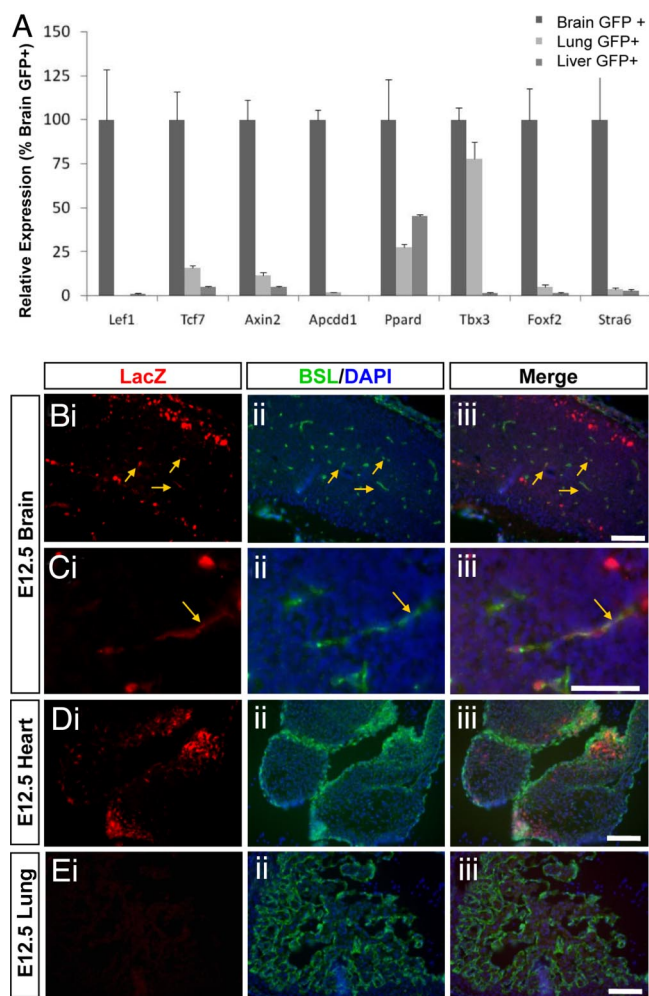
The authors declare no conflict of interest.

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**Fig. 1.** Wnt signaling is activated specifically in brain endothelial cells. (A) GeneChip analysis of Wnt signaling components in purified endothelial cells. FACS analysis was used to purify endothelial cells from the brain, liver and lung of Tie2GFP mice, and gene expression was analyzed using Affymetrix microarray analysis. The expression of several molecules that have been demonstrated to be downstream of Wnt/ $\beta$ -catenin signaling are enriched in brain endothelial cells compared with the liver and lung samples. For each probe set values are normalized to brain endothelial cells sample. (B-E) Tissue sections from the cerebral cortex (B and C higher magnification), heart (D) and lung (E) of an E12.5 TOP-Gal transgenic mouse were stained with an anti-LacZ antibody to indicate Wnt activity (i), the vessel marker BSL and the nuclear stain DAPI (ii). In merged images (iii), yellow arrows point to co-localization of LacZ and BSL signals. Wnt activity is observed in blood vessels in the brain, but not heart or lung at E12.5. [Scale bar, 100  $\mu$ m (B, D, E) and 50  $\mu$ m (C).]

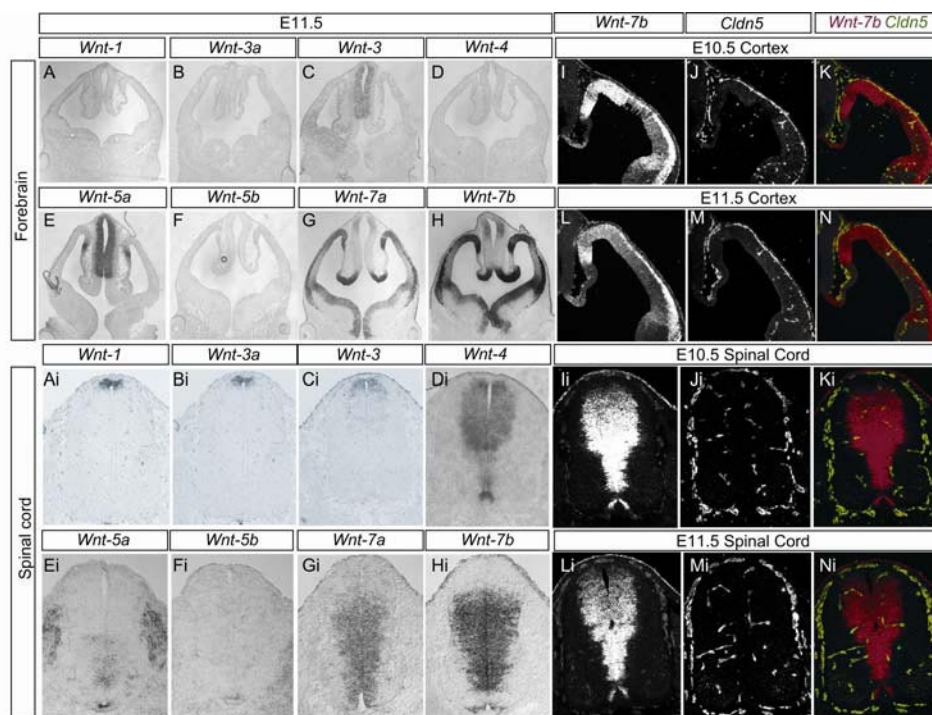
perineural vascular plexus invade the underlying neural tissue. We therefore double stained tissue sections of E12.5 TOP-Gal mice with an anti-LacZ antibody and the vascular marker *Bandeiraea simplicifolia* lectin I (BSL) (Fig. 1). We observed activated Wnt signaling, as evidenced by anti-lacZ immunostaining, in many different tissues during embryogenesis. LacZ expression, however, co-localized with the vascular marker BSL only in the CNS, but not in peripheral tissues including the heart, liver, and lung (Fig. 1 B-E). These data demonstrate that Wnt signaling is specifically activated in CNS blood vessels during development.

**Active Wnt/ $\beta$ -Catenin Signaling in the CNS Vasculature Correlates with Wnt Expression in Neural Progenitors and Frizzled Expression in Blood Vessels.** To identify which Wnt ligands may signal to CNS vessels, we next performed in situ hybridization studies using probes for

various Wnt ligand mRNAs. We found that several canonical Wnt ligands were expressed by neural progenitors in the ventricular zone of the developing mouse CNS. In particular, vascular Wnt activation temporally correlated with the expression of Wnt7a and Wnt7b in the developing forebrain and in the ventral and intermediate spinal cord; Wnt 4 in the dorsal and intermediate spinal cord; and Wnt 1, Wnt 3, and Wnt3a throughout the dorsal neural tube (Fig. 2). As our analysis of TOP-GAL Wnt reporter mice demonstrated Wnt/ $\beta$ -catenin activity in endothelial cells throughout the CNS, this reporter activity is likely activated by different Wnt ligands expressed in spatially distinct regions of the CNS. In addition, neural progenitor cells in some CNS regions also expressed Wnt ligands that act through non-canonical signaling, including Wnt 5a and Wnt 5b (Fig. 2). These ligands may also be able to activate canonical  $\beta$ -catenin signaling depending on the Frizzled receptor type(s) expressed by the endothelial cells (14). Conversely, CNS endothelial cells express the Wnt receptors Frizzled 4, Frizzled 6, and Frizzled 8, as identified by microarray analysis of purified endothelial cells (supporting information (SI) Fig. S1). Frizzled 6 expression is highly enriched in CNS endothelial cells compared to the endothelial cells of the liver and lung (Fig. S1). Analysis of Wnt7b/Claudin 5 double fluorescent in situ hybridizations demonstrate that the capillary bed is largely formed in regions of the developing forebrain and spinal cord with high Wnt7b expression (Fig. 2 I-N, Ii-Ni). Taken together, these data suggest that canonical Wnt/ $\beta$ -catenin signaling mediates endothelial-neural progenitor cellular interactions in the developing CNS.

**$\beta$ -Catenin Is Required for CNS Vessel Formation in Vivo.** To determine whether  $\beta$ -catenin is required for CNS vessel formation, endothelial-specific  $\beta$ -catenin knockout mice were generated by using  $\beta$ -catenin<sup>flox/flox</sup> and Tek-cre mice. Tek-cre mice express cre recombinase in endothelial cells throughout the body and therefore we used this method to delete  $\beta$ -catenin, an essential component of canonical Wnt signaling, from all vessels.  $\beta$ -catenin<sup>flox/flox</sup>; Tek-cre mice have been previously generated and die by E12.5 displaying mild patterning defects in the large vessels of the vitelline, umbilical cord, and head. The tissue capillary beds of these mutants appear normal, however, their CNS vasculature has not yet been examined (15). We therefore next examined the vascular pattern of E11.5 endothelial-specific  $\beta$ -catenin mutants ( $\beta$ -catenin<sup>flox/flox</sup>; Tek-cre genotype) and litter mate controls ( $\beta$ -catenin<sup>flox/+</sup>; Tek-cre and  $\beta$ -catenin<sup>flox/flox</sup> genotypes) by immunostaining tissue sections with an anti-CD31 antibody to label all endothelial cells. We observed an overtly normal vascular pattern in non-neural tissues, including the liver, lung, skin, jaw, and tail, in all genotypes examined, but found major vascular defects in the CNS of all mutant mice examined (Figs. 3 and Figs. S2 and S3). Virtually no capillaries formed throughout the developing forebrain and spinal cord of the mutant mice. Furthermore, the perineural vascular plexus was significantly thickened, suggesting that endothelial cells stalled in the meninges, unable to invade the CNS parenchyma (Fig. 3). In each mutant examined, we observed large malformed vessels that did invade the CNS parenchyma. These vessels, however, did not form discrete tubes or capillary networks, but instead remained as large aggregates of endothelial cells that were often associated with hemorrhage. The number and extent of these vascular malformations varied between animals and in different regions of the developing neural tube. In addition, the thickness of the neuroectodermal cell layer was significantly decreased in the mutants, leading to an increased ventricular volume. This defect is likely secondary to the vascular defects, as the conditional mutants have  $\beta$ -catenin deleted specifically within endothelial cells. The malformations consisted of multiple layers of CD31<sup>+</sup> endothelial cells, instead of single cell tubes as seen in the capillary beds of control animals. In some cases, these aggregates formed layered tubes with lumens; whereas, in other cases, they appeared as multicellular balls with no discernable lumen (Fig. S3). In many cases the aggregates





**Fig. 2.** Expression of Wnts in the developing mouse CNS. In situ hybridizations demonstrating Wnt ligand expression in the developing forebrain (A–H) and spinal cord (Ai–Hi) of E11.5 mice. Canonical Wnt ligands Wnt7a and Wnt7b are expressed by neural progenitors in the ventricular zone in the ventral-lateral spinal cord and cortical forebrain, whereas canonical Wnt ligands Wnt1, Wnt3, and Wnt3a are expressed by neural progenitors in the ventricular zone of the dorsal spinal cord and the hindbrain. Non-canonical Wnt ligands Wnt4, Wnt5a, and Wnt5b are also expressed by neural progenitors located in spatially distinct regions of the spinal cord and cortex. Double fluorescent in situ hybridizations in the developing forebrain (E10.5 I–K, E11.5 L–N) and spinal cord (E10.5 Li–Ki, E11.5 Li–Ni) with Wnt7b (I, L, Li, Li) and Claudin 5 (J, M, Ji, Mi) and merged (K, N, Ki, Ni) demonstrate that claudin 5 positive vessels vascularize Wnt7b positive regions of the developing CNS.

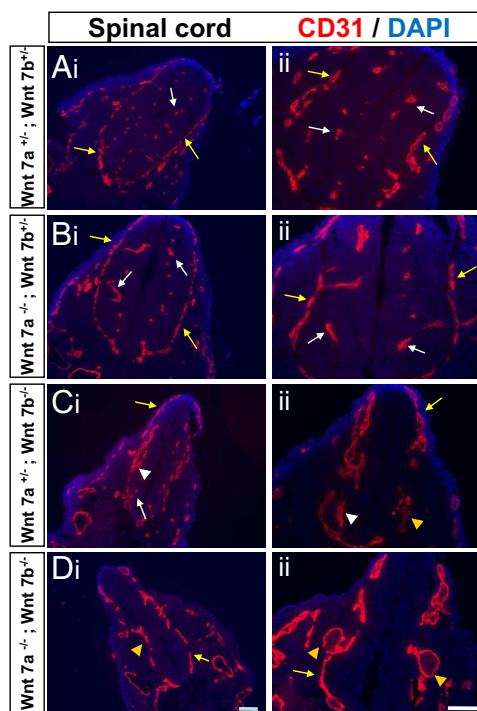
remained attached to the meninges, forming extended contacts with this vascular plexus. In most cases, the aggregates recruited pericytes, often surrounded by a layer of these mural cells (Fig. S3 A and B). The malformations were often associated with hemorrhage, which ranged from small leaks to massive bleeding into the parenchyma (Fig. S3 C and D). Interestingly, apparently normal capillary beds formed in the posterior regions of the cortex of these conditional mutants (Fig. S2), suggesting a unique mechanism for angiogenesis in this region of the CNS. Unfortunately, due to the early embryonic lethality of these mice, a complete map of  $\beta$ -catenin independent vessels remains unknown.

Taken together, the above findings demonstrate that  $\beta$ -catenin is required for the proper formation of CNS vessels, but not vessels in non-neural tissues.  $\beta$ -catenin functions not only as a transducer of Wnt signaling, but also as a component of the adherens junctions that join all endothelial cells. The vascular malformations observed in the endothelial-specific  $\beta$ -catenin mutants express other adherens junctions components at cellular junctions including  $\alpha$ -catenin,  $\gamma$ -catenin and ve-cadherin as well as tight junction components zo-1, occludin, and claudin 5 (Fig. S4, data not shown), suggesting that the defect is not due to incomplete junction protein expression. Although the expression of these proteins is unaffected, due to the cellular disorder of the malformations observed in these mice, the junctional components are also disordered. Adherens junctions connect endothelial cells in all tissues; however, the phenotype in the endothelial-specific  $\beta$ -catenin mutants is specific to the CNS matching the activation of Wnt signaling observed in the TOP-GAL mice.

**Blockade of Wnt Signaling Inhibits CNS Angiogenesis.** To further test whether the CNS-specific vascular defects in the endothelial specific  $\beta$ -catenin mutant were due to impaired Wnt signaling, and not

other functions of  $\beta$ -catenin, we next examined the consequence of delivering a Wnt inhibitor to developing embryos. In this experiment, pregnant mice at 9 days of gestation were injected with adenoviruses encoding a soluble Frizzled 8-Fc fusion (Ad-sFz8-Fc) or a control Fc (Ad-Fc). After systemic injection, adenoviruses are taken up by liver cells, which then express the molecules encoded by the viruses (16). A soluble Frizzled-8 ectodomain was used to bind extracellular Wnt ligands and Fc fusions were used to ensure delivery across the placenta. We observed many vascular malformations in the forebrains, but not non-neural tissue, of animals injected with Ad-sFz8-Fc but not when they were injected with the control Ad-Fc (Fig. S5). These malformations closely resembled those observed in the endothelial-specific  $\beta$ -catenin mutants, consisting of thickened tubes with multiple layers of endothelial cells and frequent meningeal attachment (Fig. S5 E and F). The phenotype produced by Ad-sFz8-Fc was less severe than in the endothelial-specific  $\beta$ -catenin mutants most likely because the amount of Ad-sFz8-Fc we could systemically deliver was limited by toxicity due to systemic (rather than specifically endothelial) Wnt signaling inhibition. This method inhibits Wnt-Frizzled interactions in all tissues, and thus vascular defects may be secondary to other developmental defects of inhibiting Wnt signaling. However, the similarity of the phenotype observed after Ad-sFz8-Fc injection and endothelial-specific  $\beta$ -catenin depletion, suggests that the vascular defects are due to Wnt activity on CNS endothelial cells.

**Wnt7a and Wnt7b Are Required for Normal CNS Angiogenesis.** Which Wnts regulate CNS angiogenesis? Because Wnt7a and Wnt7b have the broadest expression pattern in ventral regions of the developing CNS, we next examined the vascular pattern of Wnt7a knockout, Wnt7b knockout, and Wnt7a;Wnt7b double knockout mice. Wnt7a knockout mice are viable and exhibit a normal vascular pattern at



**Fig. 4.** Abnormal vasculature in the CNS of Wnt 7 mutants. (A–D) Coronal tissue sections of the E10.5 spinal cord in Wnt7a, Wnt7b double heterozygotes (A: Wnt7a<sup>+/-</sup>;Wnt7b<sup>+/-</sup>), Wnt7a mutants (B: Wnt7a<sup>-/-</sup>;Wnt7b<sup>+/-</sup>), Wnt7b mutants (C: Wnt7a<sup>+/-</sup>;Wnt7b<sup>-/-</sup>), and Wnt7a, Wnt7b double mutants (D: Wnt7a<sup>-/-</sup>;Wnt7b<sup>-/-</sup>) were stained with the nuclear marker DAPI (blue) and the vascular marker CD31 (red). Normal capillary beds were observed in the wild-type and Wnt7a mutants, whereas vascular malformations and thickened vascular plexus were observed in the Wnt7b mutants, and large vascular plexus dilations were observed in double mutants. Normal capillaries and normal vascular plexus are indicated with white and yellow arrows respectively, whereas vascular malformations and abnormal vascular plexus are indicated with white and yellow arrow heads respectively. (Scale bar, 100  $\mu$ m.)

esis. This is consistent with our genomic data in which several genes downstream of Wnt/ $\beta$ -catenin signaling are enriched in CNS endothelial cells compared with endothelial cells in non neural tissues (Fig. 1A). The presence of vascular malformations that fail to invade the CNS after disruption of Wnt signaling suggests that Wnt may be a potent factor stimulating migration of endothelial cells into the CNS. To test whether this is the case, we measured the ability of Wnt7a to elicit CNS endothelial cell migration in vitro across a fibronectin-coated transwell system. Indeed, Wnt7a, but not VEGF, induced a strong migration of a mouse brain endothelial cell line (bEND3.0 cells) across the filter (Fig. 5A). These results demonstrate that Wnts are a potent migration factor for CNS endothelial cells.

**Wnt/ $\beta$ -Catenin Signaling Regulates BBB-Specific Properties of CNS Endothelial Cells.** The fact that Wnt/ $\beta$ -catenin signaling regulates angiogenesis in the CNS, but not in other tissues, raises the possibility that this molecular signal imparts tissue-specific properties on the CNS endothelial cells to tightly couple CNS angiogenesis and BBB formation. CNS endothelial cells which form the BBB are characterized by the formation of tight junctions, and the expression of a variety of transporters both to provide selective transport of essential nutrients across the BBB into the brain and to efflux potential toxins from the brain. Does Wnt/ $\beta$ -catenin signaling regulate specific properties of the BBB? To test whether Wnt signaling might regulate specific components of the BBB, we next used Affymetrix microarrays to examine the transcriptional profile of purified primary CNS endothelial cells cultured in the presence





mental viability and that Wnt/ $\beta$ -catenin signaling functions to tightly couple angiogenesis to barrierogenesis. Consistent with this, Wnts have also been implicated in regulating angiogenesis in the placenta and gonads in vivo (11, 12), both tissues where blood tissue barriers are critical to their physiological function.

**Targeting Pathogenic Angiogenesis.** The present findings have several implications for understanding and treating neurological diseases. First, we observed vascular malformations after disruption of Wnt/ $\beta$ -catenin signaling that are similar to the arteriovenous malformations observed in patients with Sturge-Weber syndrome (28). Both consist of disorganized aggregates of endothelial cells that are attached to meningeal surface that hemorrhage easily. Our findings therefore have implications for understanding the origin of, and developing new treatments for, these malformations that cause debilitating leptomeningeal angiomas. Second, our findings offer new approaches for disruption of pathologic angiogenesis, without harming mature vessels. Inhibition of Wnt/ $\beta$ -catenin signaling, or downstream targets, may prevent neovascularization in diabetic retinopathy and macular degeneration or disrupt the angiogenesis that drives growth of glioblastomas and other retinal or CNS tumors (29, 30).

## Materials and Methods

**Animals.** Homozygous Tie2GFP mice (strain 003658) were obtained from Jackson Laboratory and bred to maintain homozygosity. Wnt7a<sup>+/-</sup> (strain 001253) and Wnt7b<sup>+/-</sup> (strain 004693) mice were obtained from Jackson Laboratory, and interbred to generate embryos with different combinations of mutant alleles of these Wnt 7 genes. TOP-GAL transgenic mice were provided by Roel Nusse (Stanford, CA). Conditional  $\beta$ -catenin mutant mice (strain 004152) and Tek-Cre

mice (strain 004128) were obtained from the Jackson Laboratory. Wild type C57bl6 and FVB mice were obtained from Charles River.

**Adenoviral Injections.** Adenoviruses expressing FC and soluble Frizzled 8-FC fusions were generated as previously described (16). Pregnant C57bl6 mice, 9 days gestation, were administered  $5 \times 10^8$  pfu of adenovirus via tail vein injection, and embryos were isolated 3 days later.

**In Situ Hybridizations and Immunohistochemistry.** The in situ hybridizations were performed essentially as described previously (31) with a few modifications that include incubation of fixed embryonic tissues with proteinase K for 5 min. cDNAs were either amplified from purified endothelial cells or they were obtained from Open Biosystems. Double in situ hybridizations were performed as described previously (6). For Immunohistochemistry methods see [SI Text](#).

**Migration Assays.** A mouse brain endothelial cell line (bEND3.0 cells from ATCC) was grown in DMEM, with pen/strep, sodium pyruvate, glutamate, insulin, and 10% FCS. Upon reaching confluency, the cells were starved of serum for 5 h and then trypsinized and plated at  $10^5$  cells/insert in a BD BioCoat Angiogenesis System: Endothelial Cell Migration Plate (BD Biosciences 354144) with basal media (bEND3.0 media without FCS) alone or containing 10 ng/ml VEGF (BD 354107), 0.5  $\mu$ g/ml Wnt 7a (R&D 3008-WN), or both VEGF and Wnt in the wells beneath the insert. Cells were incubated for 20 h, and then stained with 4  $\mu$ g/ml Calcein AM (BD 354216). Endothelial cell migration was calculated by determining the fluorescence in the bottom well using a fluorescence plate reader with bottom reading capabilities.

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