Canonical WNT signaling is required for proper vascularization of the CNS during embryonic development. Here, we used mice with targeted mutations in genes encoding canonical WNT pathway members to evaluate the exact contribution of these components in CNS vascular development and in specification of the blood-brain barrier (BBB) and blood-retina barrier (BRB). We determined that vasculature in various CNS regions is differentially sensitive to perturbations in canonical WNT signaling. The closely related WNT signaling coreceptors LDL receptor–related protein 5 (LRP5) and LRP6 had redundant functions in brain vascular development and barrier maintenance; however, loss of LRP5 alone dramatically altered development of the retinal vasculature. The BBB in the cerebellum and pons/interpeduncular nuclei was highly sensitive to decrements in canonical WNT signaling, and WNT signaling was required to maintain plasticity of barrier properties in mature CNS vasculature. Brain and retinal vascular defects resulting from ablation of Norrin/Frizzled4 signaling were ameliorated by stabilizing β-catenin, while inhibition of β-catenin–dependent transcription recapitulated the vascular development and barrier defects associated with loss of receptor, coreceptor, or ligand, indicating that Norrin/Frizzled4 signaling acts predominantly through β-catenin–dependent transcriptional regulation. Together, these data strongly support a model in which identical or nearly identical canonical WNT signaling mechanisms mediate neural tube and retinal vascularization and maintain the BBB and BRB.

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Canonical WNT signaling components in vascular development and barrier formation

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Introduction

In vertebrates, vascular endothelial cells (ECs) exhibit characteristic structural and functional differences among tissues (1, 2). For example, in renal glomeruli, numerous fenestrations pack the surfaces of capillary ECs, an arrangement that maximizes serum filtration (3). In contrast, in regions of the CNS with a blood-brain barrier (BBB) or blood-retina barrier (BRB), capillary ECs lack fenestrations, are joined by tight junctions, and are impermeable to serum constituents (4). Barrier competent CNS ECs express a distinctive set of plasma membrane pumps to actively extrude a wide variety of organic molecules and a distinctive set of transporters to facilitate CNS uptake of essential nutrients such as glucose. The physical and physiologic underpinnings of the BBB/BRB have been objects of fascination among biologists since their existence was first demonstrated by intravenous dye injection more than a century ago (4).

CNS vascular invasion and BBB development depend critically on canonical WNT signaling, as determined by eliminating WNT7a and WNT7b in the developing neuroepithelium and by eliminating or stabilizing β-catenin in ECs (5–7). In the retina and cerebellum, barrier integrity depends on Norrin, a TGF-β family member that is produced by glia and functions as a high-affinity WNT-like ligand for its EC receptor Frizzled4 (FZ4; refs. 8, 9). Mosaic deletion of FZ4 in mature retinal or cerebellar ECs leads to a cell-autonomous loss of the BRB or the BBB, respectively (10). Conversely, in mice lacking endogenous Norrin, initiating ectopic production of Norrin in adulthood restores BBB integrity in the cerebellum. Taken together, these data imply that BBB/BRB integrity depends on multiple canonical WNT signaling components and that this system remains plastic throughout life.

In addition to its role in CNS vascular integrity, Norrin/FZ4 signaling also plays a central role in retinal vascular growth. In mice, loss-of-function mutations in the genes coding for FZ4, Norrin, the coreceptor LDL receptor–related protein 5 (LRP5), or the integral membrane protein TSPAN12 lead to hypovascularization of the retina (8, 11–14). In humans, loss-of-function mutations in the X-linked Norrie disease gene (NDP, the gene coding for Norrin) causes severe hypovascularization of the retina, with persistence of the hyaloid vasculature, compensatory neovascularization, intraocular bleeding, retinal scarring, and blindness (15). Milder hypovascularization is associated with heterozygosity for mutations in FZD4, LRP5, and TSPAN12 (16–20) and is referred to as familial exudative vitreoretinopathy (FEVR).

In the present paper, we address the role that different WNT signaling components play in BBB/BRB integrity and in retinal vascular development. We observe a variety of anatomic patterns and severities of BBB/BRB breakdown with different combinations of mutations in genes coding for WNT signaling components, imply-
and structural similarities, LRP5 and LRP6 play substantially different roles during development: Lrp5–/– mice are viable and fertile (21), whereas Lrp6–/– fetuses have multiple developmental defects that are incompatible with postnatal life (22). In these experiments, we have used a conventional Lrp5 null allele and a conditional Lrp6 allele (23) in combination with EC- and hematopoietic stem cell–specific Cre expression (Tie2-Cre; ref. 24), and we have analyzed the phenotypes at E11.5, when CNS vascularization is well underway. At this age, no deleterious effect of gene knockout in hematopoietic cells was seen.

Eliminating both LRP5 and LRP6 in ECs (Lrp5–/– Lrp6CKO/CKO Tie2-Cre) has little effect on non-CNS vascular architecture, but led to severely attenuated CNS vascularization accompanied by cranial bleeding (Figure 1). The perineural vascular plexus lining the brain and spinal cord was hyperplastic, presumably second-

Figure 1. Effects of eliminating Ctnnb1 or Lrp5 and Lrp6 in ECs on vascularization of the E11.5 CNS. (A) Bleeding in the hindbrain, midbrain, and forebrain of a Lrp5–/– Lrp6CKO/CKO Tie2-Cre embryo at E11.5 (arrowheads, right panel). Many embryos of this genotype also show bleeding in the spinal cord. Left, control embryo. (B) Transverse Z-stacked projections at E11.5 show severe vascularization defects in the Lrp5–/– Lrp6CKO/CKO Tie2-Cre and Ctnnb1CKO/CKO Tie2-Cre hindbrain and an intermediate vascularization defect in the Ctnnb1CKO/dm Tie2-Cre hindbrain (compare the regions highlighted by the arrows). Anti-ICAM2 stains ECs more strongly outside the CNS. GS lectin binds ECs and macrophages, which invade the hypovascularized CNS. Scale bar: 200 μm. (C) Spinal cord and hindbrain vascularization defects in E11.5 embryo whole mounts imaged from the dorsal surface. Z-stacks of approximately 70-μm thickness are shown. Smooth muscle actin highlights the somites. Lrp5–/– Lrp6CKO/CKO Tie2-Cre and Ctnnb1CKO/CKO Tie2-Cre embryos (center) show severe hypovascularization, formation of glomeruloid bodies, and bleeding within the spinal cord (white). The Ctnnb1CKO/dm Tie2-Cre embryo (far right) shows an intermediate vascularization defect. Anterior (A) is at the top; posterior (P) is at the bottom. (D) Optical sections in the sagittal plane of E11.5 embryos. The top 3 panels (Lrp5+/+ Lrp6CKO/CKO Tie2-Cre, Lrp5+/– Lrp6CKO/CKO Tie2-Cre, and Lrp5–/– Lrp6CKO/CKO Tie2-Cre) show normal or nearly normal CNS vascularization. The left and middle bottom panels (Lrp5–/– Lrp6CKO/CKO Tie2-Cre and Ctnnb1CKO/CKO Tie2-Cre) show severe defects in CNS vascularization with bleeding that is most severe in the cervical spinal cord (arrowheads). The rightmost bottom panel (Ctnnb1CKO/dm Tie2-Cre) shows an intermediate vascularization defect in the spinal cord (arrowhead). Scale bar: 500 μm.

Results

Varying severities of embryonic CNS vascularization defects with loss of canonical WNT signaling components. To extend earlier observations on the defects in embryonic CNS vascularization caused by EC-specific mutation of the β-catenin gene (Ctnnb1; refs. 5–7), we examined the phenotypes caused by partially or completely eliminating EC production of LRP5 and LRP6, the 2 closely related canonical WNT signaling coreceptors. Despite their functional
ary to the failure of ECs to properly invade the neuroepithelium, and the few ECs that penetrated the neuroepithelium failed to remodel and instead formed clumps of ECs (glomeruloid bodies). This phenotype is identical to the phenotype observed with EC-specific loss of β-catenin (Ctnnb1<sup>fl/fl<sup>Cre<sup>/Cre<sup> Tie2-Cre; Figure 1, B–D). Eliminating 2 or 3 of the 4 Lrp genes copies (Lrp5<sup>−/−</sup> Lrp6<sup>−/−</sup> Ctnnb1<sup>dm</sup> TIE2Cre, Lrp5<sup>−/−</sup> Lrp6<sup>−/−</sup> TIE2Cre, or Lrp5<sup>−/−</sup> Lrp6<sup>−/−</sup> Lrp5<sup>−/−</sup> TIE2Cre) produced little or no effect on CNS vascularization, indicating that a single copy of either Lrp5 or Lrp6 suffices to support CNS vascular development at this age. We also tested the activity of a β-catenin derivative in which the transcriptional activation domain had been selectively disrupted (abbreviated “double mutant” [dm]; Ctnnb1<sup>dm</sup>; ref. 25). Earlier work suggested that this derivative retains the capacity to associate with E-cadherin, and therefore it allows a distinction to be made between β-catenin’s transcriptional and cell-junctional roles (25). At E11.5, Ctnnb1<sup>fl/fl<sup>Cre<sup>/Cre<sup> Tie2-Cre embryos showed a similar, although less severe, CNS vascularization defect compared with that seen in Ctnnb1<sup>fl/fl<sup>Cre<sup>/Cre<sup> Tie2-Cre or Lrp5<sup>−/−</sup> Lrp6<sup>−/−</sup> TIE2Cre embryos (Figure 1, B–D), suggesting either that the Ctnnb1<sup>dm</sup> allele retains some transcriptional activity and/or that β-catenin’s cadherin-binding activity plays a minor role in embryonic CNS angiogenesis. Phenotypes produced by the Ctnnb1<sup>dm</sup> allele at later times in development are described below. Table 1 provides a summary of the genotypes and phenotypes shown throughout the paper.

Widespread but nonuniform BBB defects in the adult brain with loss of Ctnnb1 or Lrp5 and Lrp6 in ECs. To extend the comparison between β-catenin and LRPs/LRP6 deficiency in ECs to the postnatal CNS vasculature, we used a Pdgfb-CreER transgene (26) to recombine the conditional alleles selectively in ECs after treatment with 4-hydroxytamoxifen (4HT). To incorporate a functional measure of BBB integrity, we examined leakage of the low molecular weight lysine-reactive biotinylation reagent sulfo-N-hydroxy-succinimide–biotin (sulfo-NHS-biotin) from the intravascular space into the brain parenchyma. WT, Ctnnb1<sup>fl/fl<sup>/Cre<sup> Pdgfb-CreER, and Lrp5<sup>−/−</sup> Lrp6<sup>−/−</sup> Pdgfb-CreER brains were analyzed at P23–P24, 1 week after 4HT treatment, or at P10, 2 days after 4HT treatment. (Early and efficient postnatal deletion of Lrp5 and Lrp6 or Ctnnb1 in ECs led to death within approximately 3 days, thereby limiting the time window of the experiment and/or the 4HT dose that could be applied.) In WT controls, extensive leakage of sulfo-NHS-biotin occurred in and around the choroid plexus, the meninges, and the ventral hypothalamus, but the remaining brain parenchyma was protected by the BBB. In contrast, Ctnnb1<sup>fl/fl</sup>/Cre<sup>/Cre<sup> Pdgfb-CreER and Lrp5<sup>−/−</sup> Lrp6<sup>−/−</sup> Pdgfb-CreER brains showed widespread leakage in the striatum (arrows in Figure 2, A, F, and K), the paraventricular hypothalamic (arrows in Figure 2, B, G, and L), the ventral thalamus and cortex (arrows in Figure 2, C, H, and M), the pons and interpeduncular nuclei (arrows in Figure 2, D, I, and N), and the cerebellum and ventral brainstem (arrows in Figure 2, E, J, and O). Mural cell coverage was examined by antibody staining against PDGFRβ and appeared essentially normal in affected regions (data not shown), suggesting an EC-autonomous basis for the permeability defect. Brain regions with BBB defects also showed changes in EC protein composition consistent with a loss of barrier function. In particular, there was induction of plasmalemma vesicle-associated protein (PLVAP), a component of endothelial fenestrations (27), which is normally absent from CNS ECs, and repression of Claudin5, a tight junction protein. As seen in Figure 2, P–V, in regions with a leaky BBB, brain regions showed widespread leakage in the striatum (arrows in Figure 2, A, F, and K), the paraventricular hypothalamic (arrows in Figure 2, B, G, and L), the ventral thalamus and cortex (arrows in Figure 2, C, H, and M), the pons and interpeduncular nuclei (arrows in Figure 2, D, I, and N), and the cerebellum and ventral brainstem (arrows in Figure 2, E, J, and O). 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These regional differences could reflect differences in the level of Pdgfb-CreER expression, accessibility of loxP targets, and/or the responses of ECs to loss of β-catenin or LRP5 and LRP6.

We next asked whether LRP5 and LRP6 act redundantly in the postnatal brain vasculature and whether a single functional Lrp allele (instead of the usual 4 alleles) could support BBB integrity. As seen in Figure 2V, Lrp5+/– Lrp6lox/lox Tie2Cre and Lrp5–/– Lrp6lox/lox Tie2Cre brains were vascularized normally with only rare foci of BBB breakdown that colocalize with sites where one or several capillary ECs had converted from a PLVAP–Claudin5+ to PLVAP+Claudin5+ or PLVAP+Claudin5–. These regional differences could reflect differences in the level of Pdgfb-CreER expression, accessibility of loxP targets, and/or the responses of ECs to loss of β-catenin or LRP5 and LRP6.

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to a PLVAP-Claudin5 state. The extreme rarity of the converted EC clusters — on average several clusters per 120-μm-thick sagittal brain section — and the likely clonal relationship of the several ECs within each cluster suggest the possibility that each cluster arose from a rare somatic genetic or epigenetic event such as loss or inactivation of the single active Lrp allele. The plausibility of this conjecture rests on well-described mechanisms leading to rare somatic loss of tumor-suppressor gene function in premalignant cells (28). We conclude that a single copy of either Lrp5 or Lrp6 suffices to support postnatal vascular development and barrier integrity in the brain.

Distinctive anatomic distributions and severities of BBB defects in the postnatal brain with different combinations of Fz4, Ctnnb1, Lrp5, and Ndp mutations. In earlier work, we observed that a generalized loss of Fz4 or Norrin impairs vascular development and BBB integrity in the retina, cerebellum, and olfactory bulb, but not in the cerebral cortex or thalamus, and we and others observed that loss of LRP5 impairs vascular development only in the retina, suggesting that different CNS regions, different combinations of Frizzled ligands (Norrin, WNT7a, WNT7b, etc.) and/or Frizzled receptors and coreceptors mediate canonical WNT signaling to promote vascular development and BBB integrity (10, 21). Defining the extent and anatomic distribution of BBB responses to perturbations in canonical WNT signaling could provide general insights into molecular and cellular heterogeneity within the CNS vasculature. As described above for Lrp5, Lrp6, and Ctnnb1, our approach has been to reduce or eliminate various combinations of canonical WNT-signaling components and then determine the territories in which BBB integrity is compromised, as judged by leakage of sulfo-NHS-biotin, induction of PLVAP expression, and loss of Claudin5 expression.

Since Fz4 is expressed in all or nearly all CNS and non-CNS ECs (9), we asked whether a wider role for Fz4 in BBB integrity could be revealed by eliminating Fz4 on a genetic background that was sensitized by partial or complete loss of Lrp5 or Ctnnb1. Eliminating 1 copy of Fz4 in combination with Lrp5 (Fz4<sup>CKO</sup>/Lrp5<sup>–/–</sup> Tie2Cre; Figure 3A), 1 copy of Ctnnb1 in combination with Lrp5 (Lrp5<sup>–/–</sup> Ctnnb1<sup>1CKO</sup>/– Tie2Cre; Figure 3F), or Lrp5 alone (Lrp5<sup>–/–</sup>; data not shown) had no apparent effect on BBB integrity. However, eliminating both copies of Fz4 in combination with Lrp5 (Fz4<sup>CKO</sup>/Lrp5<sup>–/–</sup> Tie2Cre; Figure 3, B and E) greatly expanded the territories of BBB breakdown relative to those seen with elimination of Fz4 alone (Figure 3C). In particular, P10–P11 Fz4<sup>CKO</sup>/– Lrp5<sup>–/–</sup> Tie2Cre mice showed zones of BBB breakdown in the thalamus, brain stem, cortex, and pons/interpeduncular nuclei (arrows in Figure 3, B and E). A somewhat different anatomic pattern was seen in Fz4<sup>CKO</sup>/Ctnnb1<sup>1CKO</sup>/– Tie2Cre mice: BBB breakdown was prominent in the pons/interpeduncular nuclei and cerebellum, but was not observed in the cortex, thalamus, or brain stem (Figure 3D).

In these experiments BBB loss was tightly correlated with the conversion of ECs from a PLVAP-Claudin5 to a PLVAP-Claudin5 state, as noted earlier in the context of Figure 2, P–U. We also observed a left-right mirror symmetry of BBB breakdown (e.g., Figure 3E), which implies a high degree of precision in the anatomic distribution of WNT activators and/or inhibitors in CNS tissue or in the abundance and composition of WNT-signaling components in the CNS vasculature.

Consistent with the comparison between Fz4<sup>CKO</sup>/Lrp5<sup>–/–</sup> Tie2Cre and Fz4<sup>CKO</sup>/Lrp5<sup>–/–</sup> Tie2Cre brains (Figure 3, A and B), a more extensive allelic series (Fz4<sup>CKO</sup>/– Tie2-Cre, Fz4<sup>CKO</sup>/– Lrp5<sup>–/–</sup> Tie2-Cre, Fz4<sup>CKO</sup>/– Tie2-Cre, and Fz4<sup>CKO</sup>/– Lrp5<sup>–/–</sup> Tie2-Cre) showed a progressive increase in PLVAP in cerebellar ECs and a corresponding decline in cerebellar BBB integrity, with a progressive reduction in the number of functional Fz4 and/or Lrp5 alleles (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI76431DS1). A graded phenotypic effect was also seen in the context of kidney growth when Lrp5<sup>–/–</sup>/Fz4<sup>–/–</sup>, Lrp5<sup>–/–</sup>/Fz4<sup>–/-</sup>, and Lrp5<sup>–/–</sup>/Fz4<sup>–/-</sup> embryos were compared (Supplemental Figure 2), consistent with a sensitizing effect of Lrp5 loss and analogous to the kidney growth phenotypes observed previously with loss of both Fz4 and Fz8 (29). These data indicate that loss of Fz4 and Lrp5 synergize in both CNS ECs and in kidney development.

Ndp (the gene encoding Norrin) is expressed by glia throughout the CNS: in Bergman glia in the cerebellum, Muller glia in the retina, and astrocytes in other CNS regions (30). However, mutation of Ndp produces only a relatively mild loss of BBB integrity that is confined to the cerebellum and olfactory bulb (10). To explore the possibility that Norrin might function more widely — but redundantly — with other Frizzled ligands, we examined the effect of Ndp mutation on BBB integrity in genetic backgrounds that were sensitized by heterozygous loss of Fz4 or homozgyous loss of Lrp5. (Ndp is an X-linked gene. To simplify the text that follows, we refer to both Ndp<sup>+/–</sup> females and Ndp<sup>–/–</sup> males as Ndp<sup>–/–</sup>.) Ndp<sup>–/–</sup> Fz4<sup>–/–</sup> mice showed more extensive disruption of the cerebellar BBB than Ndp<sup>–/–</sup> mice, but the BBB defects did not extend to other CNS regions (Figure 3, G and H), and Ndp<sup>–/–</sup> Lrp5<sup>–/–</sup> mice showed an even greater disruption of the cerebellar BBB, with additional BBB defects in the brain stem and thalamus (Figure 3I). (As noted above in the context of Supplemental Figure 1, heterozygous loss of Fz4 or complete loss of Lrp5 cause no defects in the cerebellar BBB.) The latter observation implies a wider role for Norrin in BBB integrity than had previously been appreciated based only on the Ndp<sup>–/–</sup> phenotype.

The results of the postnatal BBB experiments are shown in semiquantitative form in Figure 4. For each of 5 brain regions (cerebral cortex, thalamus, pons/interpeduncular nuclei, brain stem, and cerebellum), the extent of BBB compromise has been scored as absent, mild, moderate, or severe. Experiments utilizing Pdgfb–CreER<sup>–</sup>-mediated recombination (Figure 2) have not been included in this figure because in those experiments the severity of BBB dysfunction depended on 4HT dose and cannot be compared directly to the severities observed with conventional null alleles or constitutively recombined conditional alleles. Figure 4 shows a broad trend in which the pons, interpeduncular nuclei, and cerebellum are most sensitive to loss of canonical WNT signaling genes, the brainstem and thalamus are of intermediate sensitivity, and the cortex is least sensitive.

Lrp5 plays a major role and Lrp6 plays a minor role in retinal vascular development. To determine the relative importance of Lrp5 and Lrp6 in retinal vascular development, we compared the anatomy, BRB integrity, and molecular phenotype of the retinal vasculature in Lrp5<sup>–/–</sup>/Lrp6<sup>–/–</sup> Tie2Cre and Lrp5<sup>–/–</sup>/Lrp6<sup>–/-</sup> Tie2Cre mice. In P12 Lrp5<sup>–/–</sup>/Lrp6<sup>–/-</sup> Tie2Cre retinas, the vascular architecture was indistinguishable from that of WT (Figure 5A). In contrast
Figure 3. Distinctive severities and locations of BBB defects in mice with different combinations of loss-of-function mutations in canonical WNT signaling components. (A-I) Representative brain sections showing sulfo-NHS-biotin leakage and Claudin5 and PLVAP immunostaining. (A and B) Sagittal sections near the midline (with boxed regions enlarged to the right). (C-I) Coronal sections. (C–F and I) Thalamus and cortex (left panels) and cerebellum and brainstem (right panels). (G and H) Cerebellum and brainstem. In C–I, the high degree of left-right symmetry in sulfo-NHS-biotin leakage implies a nonrandom distribution of BBB loss. In all sections, territories with the greatest PLVAP induction also exhibit the greatest BBB loss. Arrows highlight some of the regions with BBB breakdown. In A and B, anterior (A) is to the right and posterior (P) is to the left. Scale bars: 2 mm (low magnification), 500 μm (high magnification) (A and B); 2 mm (C–I).
In contrast, in P12 Lrp5^{-/-} Lrp6^{CKO/+} Tie2Cre retinas, there was a paucity of deep capillaries, a conversion of all ECs to a PLVAP+Claudin5– phenotype, and multiple zones of BRB breakdown (Figure 5A), a phenotype that closely resembles the Lrp5^{-/-} phenotype (12, 31). Earlier comparisons of Fz4^{-/-}, Ndp^{KO}, and Lrp5^{-/-} retinal vascular phenotypes showed that Lrp5^{-/-} retinas exhibit a slightly milder vascular invasion defect, consistent with a model in which LRP6 plays a minor role in retinal ECs (9). The observations in Figure 5A support that model and further show that in the absence of Lrp6, a single copy of Lrp5 suffices for retinal vascular development. The comparison between Lrp5^{-/-} Lrp6^{CKO/CKO} Tie2Cre and Lrp5^{-/-} Lrp6^{CKO/+} Tie2Cre phenotypes in the retina stands in sharp contrast to the same comparison in the embryonic neuroepithelium and postnatal brain (Figure 1D and Figure 2V), where a single copy of...

Figure 4. Semiquantitative analysis of the locations and severities of postnatal BBB defects, based on sulfo-NHS-biotin leakage, with different combinations of loss-of-function mutations in canonical WNT signaling components. (A) BBB defects were scored as absent (0), mild (1), moderate (2), or severe (3) in the cerebral cortex, thalamus, pons/interpeduncular nuclei, brain stem, and cerebellum. Each circle represents the assessment for a single mouse for each of the 5 indicated brain regions. Only brains from P10 or older mice were scored. CC, cerebral cortex; T, thalamus; P/IN, pons/interpeduncular nuclei; B, brainstem; CE, cerebellum. (B) Color-coded summary of mean BBB defect scores (from part A) for each brain region and genotype. The colors represent the severity of the BBB defect, as summarized by the 0 to 3 leakage score described for A.
This alternative explanation, at least as applied to CreER expression, is unlikely because we have measured the 4HT-induced Cre activity of Pdgfb-CreER at P14–P16 using a Cre-activated nuclear tdTomato reporter knocked into the Hprt locus \[HprtLSL–tdT]\; ref. 32 and observed a uniformly high efficiency of recombination in arterial, capillary, and vein ECs \[data not shown\].

With increasing age, 4HT treatment of mature retinas converted fewer ECs to a PLVAP+Claudin5– state. This age-dependent decline could reflect a decline in accessibility of \(\text{loxP}\) targets with increasing age (a trend that is seen with many conditional alleles), or alternately it could represent a progressive decline in the plasticity of the EC state. The converted ECs were confined to veins and capillaries, where they were associated with sulfo-NHS-biotin leakage (Figure 5C). Cre-mediated recombination of Ctnnb1CKO/CKO after P15 had no obvious effect on retinal vascular architecture. The conversion of some adult retinal ECs to a PLVAP+Claudin5– state provides further evidence for ongoing canonical WNT signaling in the mature CNS vasculature.
Figure 6. Vascular growth and BRB defects in retinas with different combinations of loss-of-function mutations in canonical WNT signaling components. (A) PLVAP expression in capillary and vein ECs in Fz4+/− but not Fz4+/+ retinas. Scale bar: 200 μm. (B) Mosaic recombination in Fz4+/− Ctnnb1+/− Tie2-Cre retinas: recombined territory (upper two-thirds) with enhanced PLVAP and reduced Claudin5 in veins and capillaries and reduced vascular density, and unrecombined territory (arrows) with PLVAP–Claudin5 + ECs and normal vascular density. Scale bar: 500 μm. (C) More severe vascular defects in Fz4CKO/– Ctnnb1CKO/+ Tie2-Cre (right 4 panels) compared with Fz4CKO/– Tie2-Cre (left 2 panels). Scale bar: 500 μm. (D) Representative flat-mount retina from an adult Ndp+/− female. Rare PLVAP+Claudin5− ECs (arrows) are associated with sulfo-NHS-biotin leakage. Boxed regions in low-magnification panels are enlarged to the right. Scale bar: 500 μm. (E) Compared with Ndp+/− retinas (e.g., D), approximately 50% of adult Fz4+/− Ndp−/− retinas show more extensive conversion of ECs from to a PLVAP−Claudin5− state. The Hprt−/− reporter and the Ndp− allele are on the same X chromosome, and the Ndp− allele is on the unmarked X chromosome. tdTomato shows the pattern of X chromosome mosaicism. Retina 3318 shows few PLVAP−Claudin5− ECs. Retina 3329a shows multiple territories with a high density of PLVAP−Claudin5− ECs. Boxed regions marked a (low density of Ndp− cells) and b (high density of Ndp− cells) are enlarged at right. Scale bars: 1 mm (left panels); 200 μm (right panels).
tions of WNT-signaling components. Phenotypes associated with loss of 1 Fz4 allele are of special interest because heterozygous loss of Fz4 is a common cause of FEVR (19). Although the Fz4+/− retinal vasculature was architecturally normal, a minority of capillary and vein — but not arterial — ECs had converted to a PLVAP Claudin5 state (Figure 6A). In contrast to the retina, a single copy of Fz4 was sufficient to suppress PLVAP expression throughout the brain vasculature (data not shown). A retinal and brain EC phenotype nearly identical to that seen in Fz4+/− mice was observed in Ctnnb1+/− mice (data not shown). The variable conversion of retinal ECs implies that halving the Fz4 or Ctnnb1 gene dosage brings retinal ECs close to a threshold for conversion between mutually exclusive developmental states, one PLVAP Claudin5+ and the other PLVAP Claudin5−. This represents the first observation, to our knowledge, of a phenotypic alteration in an animal model with a genotype that matches one of the genotypes responsible for FEVR. It suggests that conversion of human retinal ECs to a PLVAP Claudin5− state may represent the fundamental cellular defect in FEVR.

Figure 7. Production of stabilized β-catenin in EC rescues Ndp−/− and Fz4+/− cerebellar BBB defects. (A) BBB integrity assessed by i.p. injection of Evans blue at P50. Brains were collected 1 day after Evans blue injection. Fz4+/− Ctnnb1flex3/− Pdgfb-CreER mice received 4 i.p. injections of a total of 330 μg 4HT/injection between P10 and P45. (B) Cerebellar BBB integrity assessed by sulfo-NHS-biotin leakage in Fz4+/− and Ndp−/− mice (left panels) and Fz4+/− Ctnnb1flex3/− Pdgfb-CreER and Ndp+− Ctnnb1flex3/+ Pdgfb-CreER mice (right panels). β-Catenin stabilization rescues the cerebellar BBB defect. In B−E, mice carrying the Ctnnb1flex3 Pdgfb-CreER allele received 3 to 4 i.p. injections of 20 to 100 μg 4HT/i injection at intervals of 2 to 3 days between P2 and P10, a regimen that leads to nearly complete EC recombination of the Ctnnb1flex3 allele in the presence of Pdgfb-CreER. Scale bar: 1 mm (B). (C and D) Neuronal death, measured by cleaved caspase 3 immunostaining, is abundant in the Fz4+/− cerebellum and is suppressed in the Fz4+/− Ctnnb1flex3/+ Pdgfb-CreER cerebellum at P18. Box and whisker plot parameters are described in Methods. Scale bar: 200 μm. (E) BBB integrity assessed by extravasation of mouse IgG and by sulfo-NHS-biotin leakage in WT, Fz4+/−, and Fz4+/− Ctnnb1flex3/− Pdgfb-CreER cerebella at P19. Although β-catenin stabilization largely rescues the cerebellar BBB defect, rare sites of leakage remain (arrows, right panels). Scale bar: 200 μm.
Although retinas heterozygous for loss of Fz4 or Ctnnb1 show little or no change in vascular architecture, when the 2 mutations were combined, the retinal vascular architecture was clearly abnormal. Figure 6B shows an unusual and especially informative Fz4^{−/−}/Ctnnb1^{−/−}/Tie2Cre retina with variegated expression of Tie2Cre. In the phenotypically WT territory (arrows), either Fz4^{−/−} or Ctnnb1^{−/−} or both have failed to recombine, and, as a result, the vasculature is architecturally normal and ECs are PLVAP Claudin5. The remainder of the image shows the typical Fz4^{−/−}/Ctnnb1^{−/−}/Tie2Cre retinal vascular phenotype, with reduced vascular density and partial conversion of many ECs to a PLVAP Claudin5 state.

If Fz4 were the only receptor responsible for canonical WNT signaling in retinal ECs, then one would predict that the Fz4^{−/−} phenotype in the retina would be indistinguishable from the most severe phenotype produced by loss-of-function mutations in canonical WNT-signaling components. To test this idea, we asked whether eliminating Fz4 and simultaneously halving the level of β-catenin leads to a retinal vascular phenotype that is more severe than that seen in Fz4^{−/−} mice. An affirmative answer would imply that Frizzled family members other than FZ4 also play a role in retinal vascular development. Figure 6C shows a comparison between Fz4^{−/−}/Ctnnb1^{−/−}/Tie2-Cre and Fz4^{−/−}/Ctnnb1^{−/−}/Tie2-Cre retinal vascular phenotype (vertical arrows in Figure 6C). By way of comparison, in WT retinas, the vasculature completely covers the retina by approximately P8 (data not shown). In some Fz4^{−/−}/Ctnnb1^{−/−}/Tie2-Cre retinas at approximately P12, the periphery is densely vascularized by an in-growth from the hyaloid vasculature (horizontal arrows in the P12 retina in Figure 6C), a pattern that is only rarely seen in Fz4^{−/−} retinas. The large-diameter PLVAP Claudin5 hyaloid vessels originate at the optic disc, grow radially over the surface of the retina, and form a dense arbor in the region of peripheral retina that is not covered by the intrinsic retinal vasculature. This growth of hyaloid-derived vessels likely reflects a response to severe hypoxia in the retinal periphery. In the P45 retina shown in Figure 6C, a fully recombined territory (horizontal arrows) with PLVAP Claudin5 ECs and a high-surface vascular density — presumably a secondary response to retinal hypoxia — is contrasted with the adjacent unrecombined territory, which is populated by PLVAP Claudin5 ECs at normal vascular density. In summary, the greater severity of the Fz4^{−/−}/Ctnnb1^{−/−}/Tie2-Cre retinal vascular phenotype compared with the Fz4^{−/−}/Tie2-Cre phenotype suggests that additional receptors — presumably other Frizzled family members — may play at least a minor role in canonical WNT signaling in retinal ECs.

Retinal vascular phenotypes in Ndp and Fz4 double heterozygotes. The X-chromosomal location of Ndp provides an opportunity to examine variation in Norrin action in Ndp^{−/−} females due to variation in the topography of X chromosome inactivation (XCI). In female mouse retinas, contiguous territories that express one or the other X chromosome generally vary from tens to hundreds of microns in diameter (32, 33). In earlier work with Ndp^{−/−} females, in which the topography of the XCI mosaic was visualized with a constitutively expressed X-linked tdTomato reporter (Hprt^{−/−}), we observed a subset of retinas with unusually coarse XCI mosaicism in which PLVAP Claudin5 vasculature showed a high spatial correlation with regions of retinal parenchyma expressing the Ndp^{−/−} allele (32).

To extend the observations of Wu et al. (32), we examined retinas that combined heterozygosity for Ndp with a sensitized genetic background in which Fz4 gene dosage was halved (Ndp^{−/−}/Fz4^{−/−}). Typically, Ndp^{−/−} retinas exhibit only a few highly localized regions of BRB deficiency, and these correspond to the locations of small numbers of ECs — or even single ECs — that are PLVAP Claudin5 (Figure 6D). In contrast, in approximately half of the Ndp^{−/−}/Fz4^{−/−} retinas, there were large numbers of PLVAP Claudin5 ECs that covered contiguous territories of up to several hundred microns (Figure 6E), a far greater size and density of PLVAP Claudin5 ECs than were found in Fz4^{−/−} retinas or in Ndp^{−/−} retinas (compare Figure 6E, panel a, with Figure 6A). In Ndp^{−/−}/Fz4^{−/−} retinas, PLVAP Claudin5 ECs were present in regions with the lowest density of retinal cells expressing the Ndp (i.e., Hprt^{−/−}) X chromosome. These data imply that in Ndp^{−/−} mice, Norrin levels in many regions of the retina are only marginally above the threshold for maintaining ECs in a PLVAP Claudin5 state, and this near-threshold level is revealed by halving the FZ4 level. If the same situation holds in humans, then genetic background differences that modulate canonical WNT signaling might be expected to produce phenotypic heterogeneity among NDP carrier females.

β-catenin stabilization rescues brain and retinal vascular defects caused by mutation of Fz4 or Ndp. Norrin shows no sequence homology or structural similarity to the WNT family of proteins (34, 35). However, in transfected HEK293 cells, Norrin, FZ4, LRP5, and Tspan12 activate canonical WNT signaling (8, 13), and the general assumption in the field has been that the roles of these proteins in retinal vascular development involve only this pathway (14). In addition to the canonical WNT pathway, 2 noncanonical pathways are also able to transmit signals from Frizzled receptors and could potentially play a role in EC development and homeostasis: the WNT/Ca++ pathway, which is hypothesized to involve Frizzled activation of a G-protein (36), and the planar cell polarity (PCP) pathway, which relays signals from asymmetrically localized cell-surface complexes to the cytoskeleton (37). The possibility that Norrin and FZ4 signal via pathways other than the canonical WNT pathway has been raised by 2 recent studies, one suggesting that FZ4 regulates arterial development via noncanonical WNT/PCP signaling (38) and the second suggesting that, in addition to its role as a FZ4 ligand, Norrin also acts as a bone morphogenetic protein (BMP) antagonist (39).

To test the hypothesis that the canonical WNT pathway is the principal or only signaling pathway that is affected by loss of Norrin or FZ4 in vivo, we first asked whether EC induction of a canonical WNT signaling reporter transgene, Tcf/Lef-lacZ (BAT-gal; ref. 40), differed between WT and Ndp^{−/−} retinas and cerebella. Immunostaining for the nuclear-localized β-galactosidase reporter showed approximately 2-fold and approximately 5-fold reductions in the number of β-galactosidase–positive EC nuclei in P4–P5 Ndp^{−/−} cerebella and retinas, respectively, a difference that was easily detected despite the substantial variegation in transgene expression (Supplemental Figure 4). Next we asked whether artificially stabilizing β-catenin in ECs could rescue the CNS vascular phenotypes in Fz4^{−/−} and Ndp^{−/−} mice. As β-catenin is downstream...
of the Norrin/FZ4/LRP complex at the plasma membrane and is the sole conduit of the canonical WNT signal from cytoplasm to nucleus, and as β-catenin stabilization appears to play no role in noncanonical WNT signaling or BMP signaling, this experiment should provide a clear test of the hypothesis. In these experiments, β-catenin stabilization was effected by Cre-mediated deletion of Ctnnb1 exon 3, which codes for sites of GSK3 phosphorylation that promote β-catenin ubiquitination and degradation (41). We note that the amino acids coded by Ctnnb1 exon 3 appear to be dispensable for β-catenin’s transcriptional regulatory activity and also that exon 3 is a multiple of 3 nucleotides in length so that its removal does not introduce a frame shift.

As shown in Figure 7, A and B, β-catenin stabilization produced a nearly complete rescue of the Fz4−/− and NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice treated with 20 µg 4HT at P2–P4. Scale bars: 200 µm. (B) Vascular density at P16 and P40. Box and whisker plot parameters; see Methods. (C) BRB integrity assessed by sulfo-NHS-biotin leakage in adult WT, NdpKO, and NdpKO Ctnnb1flex3/+ Pdgfb-CreER retinas. NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice were treated with 20 µg 4HT at P3. Scale bar: 1 mm. (D) Retinas from NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice treated with 20 µg 4HT at P2–P3. White arrows, boundary between PLVAP–Claudin5+ and PLVAP–Claudin5− territories. Scale bars: 200 µm. (E) Flat-mount retinas from NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice treated with 1 µg 4HT at P2. Depth is color coded. Scale bar: 200 µm. (F) Whole P10 retina RNAseq from WT, NdpKO, Ctnnb1flex3/+ Pdgfb-CreER, and NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice treated with 20 µg 4HT at P3. Upper panel, ratios of transcript abundances: NdpKO Ctnnb1flex3/+ Pdgfb-CreER versus NdpKO (y axis) and WT versus NdpKO (x axis). Least squares regression line (blue; R² = 0.62) and orthogonal (Deming) regression (red; ref. 69). Lower panel, ratios of transcript abundances: Ctnnb1flex3/+ Pdgfb-CreER versus WT (y axis) and WT versus NdpKO (y axis). The upper left point is likely an artifact and was not included in the line fitting, and the upper right point was also omitted from the line fitting so that it would not dominate the regression calculation. (G) Peripheral retinas of WT and Ctnnb1flex3/+ Pdgfb-CreER mice treated with 50 to 100 µg 4HT at P2–P4. Scale bars: 200 µm. (H) Left panel, horizontal eye position during OKR representative recordings. Right panel, OKR quantification. n = 3 or more mice per genotype.

When NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice were given a very low P15 (“late β-catenin rescue”) — an age when vascular invasion is well under way in WT retinas and is severely retarded in NdpKO retinas — resulted in nearly complete rescue of the inner plexiform layer (IPL) plexus but only partial rescue of the OPL plexus (Figure 8B). Finally, treatment with 4HT at P21–P25, after the completion of retinal vascular development, resulted in partial restoration of Claudin5 expression in veins and capillaries and a large increase in the number of intraretinal glomeruloid structures, which did not produce an intraretinal capillary plexus (Supplemental Figure 5). Thus, the extent of anatomic rescue depends in a graded manner on the timing of the canonical WNT signal.

Interestingly, in adult NdpKO Ctnnb1flex3/+ Pdgfb-CreER retinas treated with 4HT at P3, the central approximately 1 mm of each radial vein displayed the NdpKO phenotype, both molecularly and functionally: PLVAP was expressed, Claudin5 was repressed, and intravascular sulfo-NHS-biotin leaked through the endothelium (Figure 8, C and D). We ascribe this distinctive topography to 2 factors: the radial migration of ECs during normal retinal development and the retarded migration and delayed proliferation of ECs that lack Norrin/FZ4 signaling (9, 10). If, as seems likely, CreER-mediated recombination of the Ctnnb1flex3 allele was incomplete after a single dose of 4HT, then the early postnatal NdpKO Ctnnb1flex3/+ Pdgfb-CreER retinal vasculature would have consisted of a mixed population of defective and rescued ECs. Since the rescued ECs would be expected to proliferate and migrate more efficiently than the defective ECs, the result would be a mature retinal vasculature in which the periphery is populated largely or exclusively by rescued ECs. The data in Figure 8, C and D, indicate that ECs that lack Norrin/FZ4 signaling are at a competitive disadvantage with respect to both proliferation and migration.

A variation on the preceding Ctnnb1flex3 rescue experiment revealed the invasive properties of small numbers of rescued ECs. When NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice were given a very low dose of 4HT on or before P3 (“early β-catenin rescue”), we observed vascular invasion of the retina, the formation of a capillary plexus on either side of the inner nuclear layer (INL), and a restoration of the PLVAP–Claudin5− EC state (Figure 8A). When these retinas were examined at P16, the vascular anatomy differed from that of the WT only by a small reduction in capillary density in the outer plexiform layer (OPL) (Figure 8B). Treatment with 4HT at P10–
Figure 10. Transcriptionally inactive β-catenin cannot support normal retinal vascular development and BBB/BRB integrity. (A and B) Brains from P10 Ctnnb1^{Pten+/+} (control) and Ctnnb1^{Pten-/-} Pdgfb-CreER mice treated with 60 μg 4HT at P6. Right panels, coronal sections at the anterior hippocampus (left), the pons (center), and the cerebellum (right). Enlarged images (left) show Claudin5 and PLVAP in the cerebral cortical vasculature, corresponding to the white rectangles (center). Ctnnb1^{Pten-/-} brains show PLVAP Claudin5+ vasculature and no sulfo-NHS-biotin leakage. Ctnnb1^{Pten-/-} Pdgfb-CreER vasculature shows many PLVAP Claudin5+ ECs and extensive sulfo-NHS-biotin leakage. Scale bars: 500 μm (left panels); 2 mm (right panels). (C) Flat-mount retinas from P10 Ctnnb1^{Pten+/+} Pdgfb-CreER, Ctnnb1^{Pten-/-}, and Ctnnb1^{Pten-/-} Pdgfb-CreER mice treated with 50 to 100 μg 4HT at P6. Upper row, the BRB is compromised in Ctnnb1^{Pten-/-} Pdgfb-CreER retinas. Bottom row, retinal vasculature color coded by depth. Ctnnb1^{Pten-/-} Pdgfb-CreER retinas have far fewer deep retinal capillaries. Scale bar: 400 μm. (D) Flat-mount retinas from P10 Ctnnb1^{Pten+/+} (control) and Ctnnb1^{Pten-/-} Pdgfb-CreER mice treated with 60 μg 4HT at P6. Ctnnb1^{Pten-/-} Pdgfb-CreER retinas show efficient conversion of vein and capillary ECs from PLVAP Claudin5+ to PLVAP Claudin5-. Low levels of PLVAP in Ctnnb1^{Pten-/-} capillaries are due to heterozygosity for Ctnnb1. Scale bar: 500 μm. (E) Cross sections of P10 retinas from control Ctnnb1^{Pten+/+} Pdgfb-CreER (upper panels) and Ctnnb1^{Pten-/-} Pdgfb-CreER mice (lower panels) treated with 50 to 100 μg 4HT at P6. Ctnnb1^{Pten-/-} Pdgfb-CreER retinas show extensive vascular leakage in the IPL (arrows in the Sulfo-NHS-biotin panel) and lack deep retinal capillaries (arrows in the GS-lectin panels). Scale bar: 200 μm.
dose (~1 μg) of 4HT at P2–P4 and their retinas examined at P18, multiple well-separated clusters of ECs were found within the retina from the vitreal surface to the IPL and OPL (Figure 8E). It seems likely that each cluster arose from a single rescued EC and that this cell and its progeny were able to carry out the normal vascular invasion program.

The transcriptional response to Norrin/FZ4 signaling has been studied previously by analyzing whole retinas and immunofluorescence of vascular cells (9, 31). To assess the extent to which β-catenin stabilization in the NdpKO background restored normal EC gene expression, total retina RNA from P10 WT, NdpKO, Ctnnb1flex3/+ Pdgfb-CreER, and NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice was subjected to RNAseq and the abundances of brain EC-enriched transcripts - a set defined by transcriptome profiling of FACS sorted ECs (9, 31). To minimize transcriptome differences between experiments, the transcriptome to be compared was expanded to include differentially expressed transcripts - a set defined by transcriptome profiling of FACS sorted CNS vs. peripheral ECs (42) — were compared across genotypes (Supplemental Tables 1–4; RNAseq data has been deposited in the NCBI’s [GEO GSE56461]). To minimize transcriptome differences that might reflect cellular responses to 4HT exposure, all of the mice were treated with approximately 20 μg 4HT at P3. As seen in the upper panel of Figure 8F, NdpKO retinas recovered with stabilized β-catenin showed transcript abundance changes relative to NdpKO retinas that largely parallel the changes observed when WT is compared with NdpKO retinas. A smaller positive correlation was observed in comparing stabilized β-catenin versus WT and WT versus NdpKO (Figure 8F). Similar trends were seen when the transcriptome was expanded to include differentially expressed transcripts in a brain EC versus neuron/glia comparison (Supplemental Figure 6 and ref. 42). Transcripts coding for Sox17, a transcription factor previously shown to be induced in ECs by FZ4 signaling (9, 10, 43), were induced 6.6-fold in NdpKO Ctnnb1flex3/+ Pdgfb-CreER compared with NdpKO retinas and 3.2-fold in Ctnnb1flex3/+ Pdgfb-CreER compared with WT retinas. Taken together, these experiments imply that stabilized β-catenin largely restores the WT profile of the EC transcriptome to NdpKO ECs.

The small number of transcript abundance changes induced by stabilization of β-catenin on a WT background (Ctnnb1flex3/+ Pdgfb-CreER; Figure 8F), appeared to have little effect on vascular architecture in Ctnnb1flex3/+ Pdgfb-CreER retinas. The densities of all 3 layers of vasculature were close to normal at P40 (Figure 8B), although at earlier times, there was an increase in vascular density at the retinal periphery followed by an increase in empty collagen IV sleeves, an indicator of increased vessel remodeling (Figure 8G). The data suggest that homeostatic mechanisms largely compensated for the gene expression changes caused by constitutive activation of the canonical WNT signaling pathway in ECs. NdpKO and F24– mice lack an ERG b-wave and have little or no image-forming vision, as assessed by the optokinetic response (OKR) (ref. 9). To test the extent of functional rescue by β-catenin stabilization, we compared OKR responses between WT, NdpKO, Ctnnb1flex3/+ Pdgfb-CreER, and NdpKO Ctnnb1flex3/+ Pdgfb-CreER mice, all treated with 5 to 10 μg 4HT at P2–P4. Figure 8H shows that early stabilization of β-catenin largely rescued the OKR defect, consistent with the anatomic rescue of the retinal vasculature. The OKR responses of rescued mice were more variable than those of the WT controls (Figure 8H), most likely due to the variable extent of rescue of the vascular anatomy. Most or all of the “eye-tracking movements” in NdpKO mice appear to be random saccades unrelated to the moving visual stimulus; for consistency, they have been counted in the quantification in Figure 8H.

In sum, the phenotypic rescue by stabilized β-catenin supports the hypothesis that Norrin/FZ4 signaling influences retinal and cerebellar vascular development and barrier formation largely — and perhaps exclusively — via the canonical WNT-signaling pathway.

β-catenin stabilization converts the molecular properties of high permeability ECs in the choroid plexus to a more BBB-like state. Liebner et al. (5) showed that stabilizing β-catenin in prenatal mouse brain ECs in vivo or in adult mouse brain ECs in culture promotes expression of BBB markers and EC barrier properties, and Stemman et al. (6) showed that non-CNS ECs in mid-gestation embryos could be induced to express a BBB marker (glucose transporter Glut-1) by ectopic WNT5a expression. Here, we have asked whether ECs within a mature high-permeability vascular bed can be reconfigured to a more BBB-like state by inducing canonical WNT signaling. We focused on the choroid plexus because this vasculature expresses high levels of PLVAP and exhibits high permeability, essential features for the production of cerebrospinal fluid from a plasma filtrate.

To ensure that Cre-mediated recombination occurred only in response to 4HT injection, this experiment required a CreER driver free of background recombination in the absence of 4HT. As Pdgfb-CreER gives a low level of background recombination with a variety of reporters (data not shown), we constructed a VE-cadherin (Cdh5) knockin allele in which an internal ribosome entry site (IRES) was followed by a Cre open-reading frame fused on both 5′ and 3′ sides to DNA coding for mouse estrogen receptor ligand-binding domains that preferentially recognize 4HT (Mer-Cre-Mer or MCreM). The presence of 2 ER domains in the MCreM fusion protein substantially reduces background Cre-mediated recombination relative to the levels seen with the single ER domain in conventional CreER proteins, most likely because 4HT-independent recombination arises from rare proteolytic cleavage events that liberate the Cre domain from the ER domain that maintains cytoplasmic retention (44). In the Cdh5-IRES-MCreM allele, the IRES sequence reduces translation of the MCreM protein, further lowering Cre activity. Preliminary experiments showed that mice carrying both Cdh5-IRES-MCreM and a highly recombinogenic loxP reporter (Hprt1loxP; ref. 32) exhibited no reporter-expressing cells in the absence of 4HT (data not shown).

The choroid plexus develops prenatally, achieving its mature ultrastructure by the time of birth (45). When Ctnnb1flex3/+ Cdh5-IRES-MCreM mice were treated with 200 μg 4HT at P3 and the choroid plexus examined at P23, approximately 5% to 10% of capillary ECs had converted from PLVAP Claudin5 to PLVAP Claudin5, whereas age-matched controls showed a choroid plexus in which the capillary ECs were uniformly PLVAP Claudin5 (Figure 9). The low number of converted ECs reflects the low expression of MCreM from the Cdh5-IRES-MCreM locus. The mosaicism of the Ctnnb1flex3/+ Cdh5-IRES-MCreM choroid plexus provides a nice internal control in the form of unconverted ECs, and it demonstrates the cell autonomous nature of the conversion. We conclude from this experiment that differentiated capillary ECs in the choroid plexus possess a latent developmental plasticity that can be modulated by canonical WNT signaling.
Transcriptional regulation is the major mechanism by which β-catenin influences CNS vascular growth and barrier function. As noted in the second paragraph of Results, β-catenin has 2 distinct functions: it regulates transcription by associating with TCF/LEF factors and it maintains the integrity of junctional complexes by associating with the cytosolic domain of E-cadherin (46). In principal, the decrease in β-catenin destruction induced by canonical WNT signaling could affect either or both of these functions. To extend the analysis of transcriptional versus junctional roles of β-catenin beyond embryonic vascular development (Figure 1, B–D), we analyzed the structure of the postnatal vasculature when Ctnnb1<sup>−/−</sup> was the only functional Ctnnb1 allele in vascular ECs.

In Ctnnb1<sup>CKO/dm</sup> Pdgfb-CreER mice treated with 4HT at P6 and examined at P10, the BBB was severely compromised, as determined by sulfo-NHS-biotin leakage, and there was widespread conversion of brain ECs from a PLVAP–Claudin5+ as determined by sulfo-NHS-biotin leakage, and there was a modest decrease in Claudin5 expression in veins and capillaries compared with WT controls. Scale bar: 200 μm (upper panels); 200 μm (lower panels). (C) Quantification of branch points from retinal veins and arteries at P7 (left panel) and vascular density in the 3 retinal layers at P16 (right panel). *p < 0.01. (D) R26-LSL-tdt-dnTcf4/R26-LSL-tdt-dnTcf4 Pdgfb-CreER retinas (i.e., 2 alleles expressing dnTcf4) from P9–P10 mice treated with 50 μg 4HT at P3 show sulfo-NHS-biotin leakage and reduced vessel density (top panels). Pdgfb-CreER mediates nearly complete recombination at R26 as assessed by tdTomato fluorescence (bottom panels). Scale bar: 400 μm. (E) Genetic interaction between Ndp and R26-LSL-tdt-dnTcf4 with EC expression of dnTcf4. At P18, PLVAP–ECs increase in the vasculature of the Ndp<sup>−/−</sup>; R26-LSL-tdt-dnTcf4/+ Tie2-Cre cerebellum (lower panel; white arrows) compared with the Ndp<sup>−/−</sup> cerebellum (upper panel). Scale bar: 1 mm. (F) Synergistic effect of reducing or eliminating Tcf4 and expressing different levels of dnTcf4 in ECs. Flat-mount P9–P10 retinas from mice that had received 40 μg 4HT in ECs. Quantification of branch points from retinal veins and arteries at P7 (Figure 11, B and C). EC-specific activation of 2 copies of dnTcf4 produced a greater reduction in retinal vessel density and widespread BRB incompetence in the P9 retina (Figure 11D). Interestingly, at 3 months of age, retinas with this genotype showed PLVAP accumulation in veins but not in capillaries (Supplemental Figure 8B). This effect could not be attributed to differential production of dnTcf4 in the different types of blood vessels because visual inspection and quantitative analysis of pixel intensity revealed similar levels of accumulation of the cotranslated tdTomato reporter in capillaries and veins (Supplemental Figure 8B).

To further explore the role of Tcf4<sup>−/−</sup>, we examined the retinal vasculature in mice with mutations in the Tcf4<sup>−/−</sup> gene. Surprisingly, Tcf4<sup>−/−</sup>; Tie2-Cre mice showed no anatomic retinal vascular defects and no BBB defects, as judged by sulfo-NHS-biotin perfusion. However, combining a reduction in Tcf4<sup>−/−</sup> with expression of dnTcf4 synergized to produce a change in PLVAP and Claudin5 expression, as might be expected if both proteins compete for the same DNA target sites. Specifically, with each additional allele producing dnTcf4 in ECs and with each reduction in the number of endogenous Tcf4 alleles in ECs, there was a progressive increase in PLVAP and a progressive reduction in Claudin5 in retinal vein and capillary ECs at P9–P10 (Figure 11F). Similar to the effects seen with expressing dnTcf4 on a WT background, in older retinas (P30) with various combinations of dnTcf4 and Tcf4 mutations, ECs with increased PLVAP and reduced Claudin5 were confined to vein ECs (Supplemental Figure 8A). These experiments implicate TCF4 as one of the LEF/TCF family members that mediate canonical WNT signaling in ECs, but the relatively modest effect that homozgyous EC-specific loss of TCF4 had on vascular architecture implies that other family members are also active in these cells. These experiments also reveal a difference between vein and capillary ECs in their responses to perturbations in TCF4 function.

Taken together, the experiments with Ctnnb1<sup>−/−</sup> and dnTcf4 confirm and extend earlier observations (5, 6, 51), and they imply that β-catenin– and TCF/LEF-dependent transcriptional activa-
tion is the major mechanism by which canonical WNT signaling modulates retinal and brain vascular development and barrier formation and maintenance, but they leave open the possibility that nontranscriptional mechanisms may play a minor role.

**Discussion**

In this paper, we describe the responses of different subsets of developing and mature ECs to changes in the abundance of canonical WNT signaling components. These experiments reveal previously unappreciated region-specific and EC cell type–specific differences in sensitivity to perturbations in canonical WNT signaling. We also present evidence that Norrin/FZ4 signaling in the retina and cerebellum occurs predominantly or exclusively via activation of canonical WNT signaling (β-catenin stabilization) and that β-catenin mediates most of its effects on vascular development and BBB homeostasis via transcriptional regulation.

**Diversity within the brain vasculature: canonical WNT signaling thresholds and the topography of BBB homeostasis.** Beyond the universal division of all ECs into arterial, vein, and capillary subtypes, ECs that populate the bulk of the CNS vasculature have generally been viewed as a phenotypically uniform population. The exceptions to this generalization are the ECs in the vasculature of the posterior pituitary, the circumventricular organs, and the choroid plexus—specialized regions of the brain with highly permeable vessels (52). Our experiments indicate that there is another layer of phenotypic diversity among CNS ECs that can be revealed by compromising canonical WNT signaling. This diversity is especially striking in the cerebellum, where the BBB in the molecular layer is highly sensitive to a reduction in canonical WNT signaling and the BBB in the granule layer is largely resistant (Figures 2O, 3H, and 7B). These differences could reflect some combination of (a) intrinsic differences in sensitivity to canonical WNT signaling, (b) differences in the abundance or activity of Frizzled and LRP receptors in ECs, (c) differences in the local abundance or composition of canonical WNT ligands (WNTs and Norrin) and extracellular modulators of canonical WNT signaling (Dkk, Sost, and other binding proteins; ref. 53), and (d) regional variations in unrelated signal transduction pathways that could synergize with or antagonize canonical WNT signaling or that could otherwise compensate for a reduction in canonical WNT signaling. The data also imply that multiple regions of CNS vasculature have a level of canonical WNT signaling that is only marginally above the threshold for maintaining barrier integrity.

**Canonical WNT signaling in EC development and homeostasis.** The importance of WNT/FZ and Norrin/FZ signaling in CNS vascular development is now well established, but the striking similarities between embryonic neural tube and postnatal retinal vascularization defects produced by loss of canonical WNT signaling have not been widely appreciated. In both contexts, normal development involves a vascular plexus on the outer surface of the neuroepithelium that serves as a source of angiogenic ECs. In the absence of canonical WNT signaling, EC invasion is arrested near the neuroepithelial surface and the resulting clusters of ECs (glomeruloid bodies) do not produce a vascular plexus (Figure 12). The similarities between the neural tube and retinal vascularization phenotypes suggest that angiogenic ECs are using the same genetic regulatory circuits and cell biological programs in the 2 contexts. It would be interesting to extend this comparison by exploring the roles in neural tube vascularization of other signaling pathways such as Notch and Semaphorin/Plexin that have been studied in the context of retinal vascular development (54).

The roles of most of the genes that are regulated by canonical WNT signaling in ECs remain obscure. In the present experiments, 232 transcripts were observed to change more than 2-fold in WT retinas compared with Ndp*retinas, including 7 identified by Daneman et al. (42) as enriched in CNS over non-CNS vasculature. Earlier work identified and the present work confirms Sox17 as a transcription factor that is induced by Norrin/FZ4 signaling. Experiments in which constitutive production of Sox17 in Fz4*ECs in culture restored cell motility support a role for Sox17 in orchestrating the Norrin/FZ4 response (9). The diversity of EC transcripts that respond to Norrin/FZ4 signaling — including transporters, transcription factors, and enzymes — implies that canonical WNT signaling influences a wide variety of physiologic and cellular functions.

One of the most interesting features of canonical WNT signaling in ECs is its link to the plasticity of the barrier phenotype. In mature CNS ECs, activation or inhibition of canonical WNT signaling can induce or reverse the barrier state (present work; refs. 5, 10). It is interesting that after an experimentally induced reduction in canonical WNT signaling, the appearance of PLVAP is more rapid than the disappearance of Claudin5, a differential that could reflect the rapidity of transcriptional activation of PLVAP and a long half-life of Claudin5. This kinetic difference suggests that transient decrements in canonical WNT signaling might permit permeation of small molecules through capillary fenestrations without compromising EC-EC tight junctions.

**Figure 12. Schematic of angiogenesis defects in the neural tube and retina.** Top, early postnatal retina showing vascular invasion in WT versus a canonical WNT-signaling–deficient mutant (loss of Ndp or Fz4). Bottom, E11.5 neural tube showing vascular invasion in WT versus a canonical WNT-signaling–deficient mutant (loss of Ctnmb1 or Lrp5 and Lrp6). In the mutant cases, there is stunted invasion with the formation of glomeruloid bodies and hypertrophy of the surface vasculature. On the left, the currently defined components that mediate canonical WNT signaling are shown for retinal and neural tube angiogenesis.
Implications for diseases associated with BBB/BRB breakdown. Defects in Norrin/FZ4 signaling that lead to the most severe retinal hypovascularization phenotypes — Norrie disease (due to NDP mutation; ref. 15) and osteoporosis-pseudoglioma syndrome (due to homozygosity or compound heterozygosity for LRPS mutations; ref. 55) — are typically associated with little or no vision at birth. For these 2 disorders, postnatal interventions are unlikely to be efficacious. However, partial loss of Norrin/FZ4 signaling — as seen in FEVR caused by heterozygous loss of FZD4, LRPS, or TSPAN12 — is associated with a wide range of disease severities, from subclinical to severe retinal traction and detachment (16–20, 56). The finding that Fe4+− mice exhibit partial conversion of retinal ECs to a PLVAP+ state suggests that the same EC conversion may be occurring in FEVR patients. If chronically reduced levels of Norrin/FZ signaling are causally related to FEVR pathogenesis, then a therapy that modestly enhances Norrin/FZ4 signaling in the retinal vasculature might block or slow disease progression.

Among FEVR patients whose DNA has been subjected to exome sequencing, approximately half have been found to harbor mutations in TSPAN12, FZD4, NDP, or LRPS (17). Recently, Collin et al. (57) identified mutations in the gene encoding the Zinc finger transcription factor ZNF408 in 2 FEVR families, raising the number of FEVR genes to 5. The possibility that interactions among more than one disease or risk alleles may account for some of the clinical heterogeneity observed among FEVR patients who harbor the same allele was raised by Poulter et al. (20) based on their identification of homoygous TSPAN12 mutations in several patients with severe FEVR symptoms. Kondo et al. (58) have made a similar observation in a patient with severe FEVR symptoms who was homoygous for a FZD4 mutation. Our observations of multiple gene-gene interactions and low thresholds for anatomic and biochemical defects support this idea.

BBR defects are present in a wide variety of ophthalmic disorders, and BBB defects are present in a wide variety of neurologic disorders. BRB incompetence produces macular edema, and in the context of diabetic retinopathy or age-related macular degeneration, this condition can be treated by intraocular injection of anti-VEGF antibodies or soluble receptors (59). If Norrin/FZ4 signaling plays a role in regulating vascular permeability in the context of macular edema, then greater efficacy might be obtained by a combination therapy that both antagonizes VEGF and enhances Norrin/FZ4 signaling. In the brain, BBB breakdown accompanied by brain edema occurs in the context of inflammation and tissue injury, including trauma and stroke (60). It would be of great interest to determine whether this breakdown involves a decline in canonical WNT signaling in ECs.

BBR permeabilization for drug delivery. Promoting a transient disruption in the BBB for the purpose of enhancing CNS drug delivery has been an area of active investigation, but it has had limited success in the clinic. Early work focused on perfusing a hyperosmolar mannitol solution into the carotid artery to transiently shrink ECs and disrupt EC-EC junctions (61). Recent preclinical studies have shown promising results with intravascular microbubbles that absorb focused ultrasound energy to locally disrupt the BBB (62).

Using a function-blocking anti-FZ4 monoclonal antibody, Paes et al. (63) have demonstrated disruption of the BBB following systemic mAb delivery in adult mice. Extrapolating from the experiments of Paes et al. and from the genetic studies of WNT signaling in the adult CNS vasculature, it would be interesting to test whether transient pharmacologic inhibition of canonical WNT signaling could disrupt the BBB in a controlled fashion. The rapid induction of PLVAP in mouse ECs following loss of canonical WNT signaling suggests that a brief treatment with canonical pathway inhibitors might transiently induce EC fenestrations without significantly perturbing EC-EC junctions. Treatment for a longer duration would be predicted to eventually weaken or disrupt EC-EC junctions. Both short- and long-term pharmacologic blockade of WNT signaling might usefully enhance drug delivery to the CNS. We also note that pharmacologic and ultrasound/micro-bubble approaches might be synergistic. For example, if WNT signaling in CNS ECs could be transiently inhibited, the reduction in EC-EC junction stability might render the vasculature more sensitive to focused ultrasound/microbubble stress, with reduced risk for tissue injury.

Methods

Mice. The following transgenic mouse alleles were used: Ctnmb1lox (25), Ctnmb1lox (41), Lrp5 (21), Lrp6CNO (23), BAT-gal (40), Tcf4KO (64), Tie2-Cre (24), Pdgfrb-CreER (26), Fz4 (65), Fz4CNO (9), and NdpcKO (9). The R26-LSL-tdT-dnTcf4 allele was generated by inserting a cytomegalovirus/β-actin promoter/enhancer (CAG) membrane tethered tdTomato (i.e., tdTomato tagged with an N-terminal myristoylation site)– triple-myc-2A-dnTcf4 cassette at the ROSA26 locus by homologous recombination in ES cells; see Tang et al. (66) for a description of the 2A peptide technology. The dominant-negative TCF4 polypeptide consists of a methionine followed by amino acids 162–460 of TCF4 (TCF7L2) transcript variant 1 (47). The Cdh5-IRES-MCreM allele was constructed by inserting an IRES-Mer-Cre-Mer cassette (44) into the 3′ UTR of the Cdh5 gene by homologous recombination in ES cells. ES cells with the correct targeting event were identified by Southern blotting and injected into blastocysts. The resulting chimeric founders were bred using standard methods.

Antibodies and other reagents. Antibodies used in this study were as follows: rabbit anti-calbindin (Swant; CB38a); rat anti-mouse CD102/ICAM2 (BD Biosciences; 553326); rabbit anti-GLUT-1 (Thermo Scientific; RB-9052-P0), mouse anti–Claudin5 clone 4C3C2, Alexa Fluor 488 conjugate (Invitrogen; 352588); rat anti-PLVAP/MECA-32 (BD Biosciences — Pharmingen; 553849); mouse anti-α smooth muscle actin clone 1A4, Cy3 conjugate (Sigma-Aldrich; C6198), rabbit anti–Pdgfr-β (BD Transduction Laboratories; 553849); rabbit anti-calbindin (Abcam; 9361-162–460); and 488 nm streptavidin (Invitrogen; S11223). Anti–β-tubulin (Sigma-Aldrich; T5168), rabbit anti–smooth muscle actin (SMP-800), rat anti–mouse CD102/ICAM2 (BD Biosciences; 553326); rabbit anti-GLUT-1 (Thermo Scientific; RB-9052-P0), mouse anti–Claudin5 clone 4C3C2, Alexa Fluor 488 conjugate (Invitrogen; 352588); rat anti-PLVAP/MECA-32 (BD Biosciences — Pharmingen; 553849); mouse anti-α smooth muscle actin clone 1A4, Cy3 conjugate (Sigma-Aldrich; C6198), rabbit anti–Pdgfr-β (BD Transduction Laboratories; 553849); rabbit anti-calbindin (Abcam; 9361-162–460); and 488 nm streptavidin (Invitrogen; S11223).
(PBS plus 0.5% Triton X-100 plus 0.1 mM CaCl₂) plus 10% normal goat serum, washed in PBSTC for 6 to 8 hours, and incubated at 4°C overnight in secondary antibodies diluted in PBSTC plus 10% normal goat serum. The retinas were washed in PBSTC and flat mounted in Fluoromount G (EM Sciences; 17984-25). For brain vasculature, deeply anesthetized mice were perfused with PBS followed by 1% PFA in PBS. Dissected brains were immersed in cold 100% methanol at 4°C overnight and then rehydrated in PBS for 2 to 4 hours at 4°C. Brains were dissected and embedded in 3% agarose and 100- to 150-μm sections were cut on a vibratome, immunostained, and imaged. For immunostaining with rat mAbs, 0.25% to 1% mouse serum was used as a competitive blocker during incubation with the secondary antibody to lower anti-mouse crossreactivity. Whole-mount E11.5 embryos were immunostained, dehydrated, and clarified in benzyl benzoate: benzyl alcohol as described in Hua et al. (67). Images were captured using a Zeiss LSM700 confocal microscope and processed with Zen, Adobe Photoshop, ImageJ (http://imagej.nih.gov/ij/), and Adobe Illustrator software.

Arteries and veins are readily distinguished in retinal flat mounts. When compared at the same radial distance from the optic disc, arteries have a smaller diameter and are surrounded by a capillary-free zone. This assignment is confirmed by immunostaining for smooth muscle actin, which is present in perivascular cells that surround arteries but not in pericytes around veins or capillaries.

**Evans blue and sulfo-NHS-biotin injections.** For sulfo-NHS-biotin perfusion, deeply anesthetized mice were perfused with 10- to 30-ml 0.3 mg/ml sulfo-NHS-LC-Biotin (Thermo Scientific; 21335) in PBS, followed by 2% PFA in PBS. Eyes were postfixed in 1% PFA at 4°C for 4 to 6 hours prior to retina dissection. Whole-mount retinas and 100- to 150-μm vibratome sections of brain were incubated in Texas red streptavidin: Evans blue and sulfo-NHS-biotin were scored by 2 observers (Y. Wang and J. Nathans) using 4 categories for BBB breakdown: absent (i.e., indistinguishable from WT), mild, moderate, or severe. Examples of mild, moderate, and severe breakdown of the BBB in the cerebellum are seen in Figure 3, G–I, respectively.

**Quantification of vascular branching points.** Starting with images of retina flat mounts, retinal vascular branch points were counted along each radial vein or artery starting at the optic disc and terminating at a point that was 0.75 of the radial distance from the optic disc to the edge of the vascular plexus. The number of branch points was then normalized to the total length of the vessel.

**Quantification of retinal vascular coverage.** For the Ctnnb1flex3/+ experiments, 4 Z-stacked images of retina flat mounts, each covering 0.64 × 0.64 mm² and offset approximately 0.3 to 0.6 mm from the optic disc, were captured from each GS lectin-stained retina using a Zeiss LSM700 microscope. For each of the 3 vascular beds (at the vitreal surface, IPL, and OPL), the images were thresholded, binarized, and skeletonized using ImageJ. The relative vascular lengths were measured by computing the pixel coverage (which is proportional to length) of the skeletonized vessels.

**Quantification of cerebellar granule cell death.** Apoptotic cells were detected in 150-μm floating sections of cerebellum by immunostaining for anti-cleaved caspase 3. One section (from a matched cerebellar location) was counted per mouse, and 3, 6, and 8 WT, Fz4–/–, and Fz4–/– Ctnnb1flex3/+ Pdgfb-Cre mice, respectively, were analyzed.

**Quantification of β-galactosidase positive retinal and cerebellar ECs**. β-Galactosidase+ ECs were detected in whole retinas and in 150-μm floating sections of cerebellum by immunostaining with anti-β-galactosidase combined with GS lectin binding. For the retina, all β-galactosidase+ ECs were counted from flat-mount images. For the cerebellum, Z-stacked images encompassing approximately 15 μm were collapsed and the β-galactosidase+ ECs in the vasculature along the surface of the molecular layer were counted.

**Semi-quantitative scoring of BBB breakdown.** Confocal images of 100- to 150-μm vibratome sections from mice perfused with sulfo-NHS-biotin and stained with Texas red streptavidin were scored by 2 observers (Y. Wang and J. Nathans) using 4 categories for BBB breakdown: absent (i.e., indistinguishable from WT), mild, moderate, or severe. Examples of mild, moderate, and severe breakdown of the BBB in the cerebellum are seen in Figure 3, G–I, respectively.

**OKR.** OKR recordings and analysis were performed on head-fixed mice using infrared video recordings of pupil position as described in Cahill and Nathans (68). The visual stimulus was projected onto the inner walls of a vertical cylinder surrounding the head-posted mouse, and during alternating 30-second intervals, the stimulus consisted either of horizontally rotating vertical black and white stripes or a uniform gray of equivalent mean intensity, represented, respectively, by stripes and by gray zones at the top of Figure 8H.

**Statistical analysis and graphical presentations.** Box and whisker plots (RStudio) show, at their centers, the median (horizontal bar) and the 25th and 75th percentiles (box). The whiskers are located at the positions of the furthest-flung data points above or below the mean that are within a distance of 1.5 times the 25th–75th percentile distance (i.e., height of the box) either below the 25th percentile position or above the 75th percentile position. Any data points beyond the whiskers are plotted individually. For statistical analysis, an unpaired 2-tailed Student’s t test was performed, and P < 0.01 was considered significant.

**Study approval.** Mice were handled and housed according to the approved Institutional Animal Care and Use Committee (IACUC) protocol MO13M469 of the Johns Hopkins Medical Institutions. All animal studies were approved by the IACUC of the Johns Hopkins Medical Institutions.
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