Pericyte-derived vitronectin regulates blood-CNS barrier function via integrin signaling

Swathi Ayloo[1], Christopher Gallego Lazo[1], Shenghuan Sun[1,†], Wei Zhang[2], Bianxiao Cui[2], Chenghua Gu[1*]

[1] Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA

[2] Department of Chemistry, Stanford University, Stanford, California 94305, USA

[†] Current address: Biomedical Sciences Graduate Program, UCSF, San Francisco, CA 94143, USA

[*] Correspondence: chenghua_gu@hms.harvard.edu

Blood-central nervous system (CNS) barriers are physiological interfaces separating the neural tissue from circulating blood and are essential for neuronal function and cellular homeostasis. Endothelial cells that form the walls of CNS blood vessels constitute these barriers but barrier properties are not intrinsic to these cells; rather they are actively induced and maintained by the surrounding CNS microenvironment. Notably, the abluminal surface of CNS capillary endothelial cells is ensheathed by pericytes and astrocytic endfeet. However, the specific extrinsic factors from these perivascular cells that regulate barrier integrity are largely unknown. Here, we establish vitronectin, an extracellular matrix protein secreted specifically by CNS pericytes as an essential factor in regulating blood-CNS barrier function via interactions with its integrin receptor in adjacent endothelial cells. Genetic ablation of vitronectin results in leaky blood-CNS barriers, despite having normal pericyte coverage and vascular patterning. Electron microscopy reveals increased transcytosis in endothelial cells of *Vtn*$^{-/-}$ mice without functional defects in tight-junctions. We further demonstrate that vitronectin binding to integrin receptors is essential for barrier function, as mice harboring a point mutation in vitronectin that specifically abolishes integrin binding, *Vtn*^{RGE}, phenocopy the barrier defects in *Vtn*$^{-/-}$ mice. Furthermore, endothelial-specific deletion of integrin α5, an RGD-ligand binding integrin receptor that is expressed in CNS endothelial cells, also results in similar blood-CNS barrier defects as observed in *Vtn*$^{-/-}$ and *Vtn*^{RGE} mice. Finally, integrin α5 activation by vitronectin inhibits transcytosis in endothelial cells and vitronectin-integrin α5 signaling regulates barrier function independent of the caveolae pathway. These results demonstrate that signaling from perivascular cells to endothelial cells via ligand-receptor interactions is a key mechanism to regulate barrier permeability.

Summary: Vitronectin-integrin signaling between pericytes and CNS endothelial cells regulates blood-CNS barrier function

The CNS requires an optimal and tightly regulated microenvironment for efficient synaptic transmission. This is achieved by blood-CNS barriers that regulate substance flux to maintain tissue homeostasis. Two such barriers are the blood-brain barrier (BBB) and blood-retina barrier (BRB) that are physiologically similar barriers separating the blood from brain and retina, respectively. The restrictive permeability of CNS endothelial cells that constitute these barriers is a result of specialized tight junctions and low rates of transcytosis, which limit substance exchange between blood and the CNS tissue (*1–6*).

Barrier properties are not intrinsic to CNS endothelial cells, they require active induction and maintenance from the CNS environment. Early chick-quail transplantation studies demonstrated that CNS contains cues to induce barrier properties (*7*). Indeed, ablation of pericytes or astroctyes results in barrier disruption (*8–10*). These studies demonstrate the importance of these

perivascular cells in barrier function. However, the specific signals from these perivascular cells that regulate barrier properties in CNS endothelial cells are largely unknown.

Pericytes ensheath capillary endothelial cells and share the same basement membrane with them allowing for elaborate cell-cell signaling between these two cells. Intriguingly, the brain and retina have the highest pericyte to endothelial cell ratio compared to that in other tissues (*11*, *12*). Further, pericyte-deficient mice with decreased pericyte coverage around endothelial cells exhibit leaky blood-CNS barriers (*8*, *9*, *13*, *14*). These findings indicate an active role for pericytes in barrier function, suggesting that pericytes likely release factors onto endothelial cells to regulate barrier permeability. However, it is not known what specific intercellular signaling between pericytes and endothelial cells is required for this process.

Here, we identify vitronectin, a pericyte-secreted extracellular matrix (ECM) protein as an important regulator of barrier integrity. We establish that vitronectin regulates barrier function via binding to its integrin receptors on endothelial cells. Specifically, vitronectin is enriched in CNS pericytes and mice lacking vitronectin as well as vitronectin mutant mice (Vtn^{RGE}) that cannot bind integrin receptors exhibit barrier leakage. Moreover, we found that RGD-ligand binding integrins, α5 and αv are expressed in CNS endothelial cells and endothelial cell-specific acute deletion of α5 but not αv results in leaky barrier. We further demonstrate that barrier leakage observed in vitronectin mutant mice is due to increased transcytosis in CNS endothelial cells; and vitronectin-integrin α5 mediated transcytotic regulation is independent of caveolae pathway as barrier deficits we observed in $Vtn^{-/-}$ are not rescued by the genetic ablation of caveolin-1. Together, our results indicate that ligand-receptor interactions between pericyte-derived vitronectin and its integrin receptor expressed in CNS endothelial cells are critical for barrier integrity and may provide novel therapeutic opportunities for CNS drug delivery.

Vitronectin is enriched in CNS pericytes compared to pericytes of peripheral tissues and its expression coincides with functional barrier formation

In order to identify pericyte candidate genes important for barrier function we took advantage of the retina as a model system. In the retinas of mice, CNS vessels invade the optic nerve head at postnatal day (P1) and expand radially from the center toward the periphery. As vessels grow from the proximal to distal ends of the retina, proximal vessels gain barrier properties and have a functional BRB, while the newly formed distal vessels have a leaky BRB (*4*). By examination of an existing transcriptome comparing the proximal and distal retinal vessels that contain a mixture of endothelial cells and pericytes (*15*) and a brain pericyte transcriptomic database (*16*), we identified CNS pericyte genes that correlated with functional blood-retinal barrier. Of these candidate pericyte genes, we focused specifically on secreted and trans-membrane proteins as

these are likely involved in ligand-receptor interactions. These analyses identified vitronectin, an extracellular matrix protein.

To validate our gene expression analyses, we first examined vitronectin protein localization in the CNS. Consistent with enriched *Vtn* transcript in proximal compared to distal vessels of the developing retina(*15*), we also observed vitronectin protein highly expressed in proximal vessels with sealed BRB, and was not detectable in the distal, leaky vessels of the retina at P7 (Fig. 1A, Fig. S1A). Similarly, vitronectin protein was also detected along capillaries of the brain (Fig. 1B). As vitronectin is a secreted protein, to determine the cells producing *Vtn*, we used in situ hybridization to examine *Vtn* mRNA in the brain. *Vtn* mRNA is specifically expressed in *Pdgfrb* expressing pericytes adjacent to capillary endothelial cells (Fig. 1C). At P7, *Vtn* is expressed in >97% (115 out of 118 cells) of the *Pdgfrb+* pericytes abutting capillaries and was present throughout the brain (Fig. 1C, Fig. S1B). In contrast to high *Vtn* mRNA in CNS pericytes, we observed little to no expression in pericytes of peripheral tissues, such as the lung (Fig. 1D, E). Our data are consistent with recent single-cell RNA sequencing studies revealing abundant *Vtn* expression in pericytes and little to no expression in non-vascular cells such as neurons and astrocytes, both in the brain and the retina, through development and adulthood (*17–19*).

Vitronectin is required for blood-CNS barrier integrity

To determine if vitronectin is essential for barrier function, we performed a tracer leakage assay in P10 $Vtn^{-/-}$ mice when BRB and BBB are fully functional (*3*, *4*). The injected tracer was completely confined to vessels in control mice, whereas tracer leaked out of vessels in the retinas of $Vtn^{-/-}$ mice. Numerous leaky hotspots of the tracer were apparent in the parenchyma (Fig. 2A) and in neuronal cell bodies (Fig. 2B) in the retinas of these mice (Fig. 2C). This leakage was not limited to just small tracers like Sulfo-NHS-Biotin (0.5 kDa) as we observed similar leakage with higher molecular weight tracers such as 10 kDa Dextran (Fig. S2A). Furthermore, this leakage persisted through adulthood in these mice (Fig. S2B). Similar to the retina, we also observed BBB leakage in the cerebellum (Fig. 2D, E). Thus, these findings demonstrate an important role for pericyte-secreted vitronectin in blood-CNS barrier function.

Vitronectin regulates blood-CNS barrier function by suppressing transcytosis in CNS endothelial cells

To determine the subcellular basis in endothelial cells for the underlying leakage we observed in $Vtn^{-/-}$ mice, we injected horse-radish peroxidase (HRP) intravenously and performed EM analysis in retina and cerebellum. In both retina and cerebellum of $Vtn^{-/-}$ mice and wildtype littermates, we observed HRP halting at tight junction “kissing points” between endothelial cells of capillaries (Figure 2F, G), indicating functional tight junctions. Consistent with this, protein expression and localization of Claudin-5 in the retinas of $Vtn^{-/-}$ mice was also comparable to wildtype mice (Fig.

S3A). In contrast, both retinal and cerebellum endothelial cells of *Vtn*$^{-/-}$ mice exhibited significantly increased HRP-filled vesicles (Fig. 2H, I). Retinal endothelial cells had a 3-fold increase in vesicles compared to wildtype mice (Fig. 2J) and cerebellar endothelial cells had 2-fold increase (Fig. 2K), revealing that transcytosis is upregulated in these mice. Interestingly, in the cerebellum of these mice, we also observed HRP-filled vesicles in adjacent pericytes and about 25% of capillaries had their basement membrane completely filled with HRP (Fig. S3B-D). This result indicates that tracer-filled vesicles are indeed transcytosed across endothelial cells. (Example 2 of Fig. S3B). These data reveal that barrier leakage in *Vtn*$^{-/-}$ mice is due to upregulated transcytosis. Together, these observations demonstrate that pericyte-derived vitronectin regulates barrier function by specifically inhibiting transcytosis in CNS endothelial cells.

Vitronectin is not required for normal vessel patterning or pericyte coverage

How does vitronectin, a pericyte secreted protein regulate barrier properties in endothelial cells? One possibility is that barrier defects are due to impaired vessel development as pericytes are known to regulate endothelial sprouting and angiogenesis in the postnatal retinal vasculature(*20*). However, we observed no changes in vascular patterning (Fig. 3A). Specifically, we observed no changes in vessel density, capillary branching or radial outgrowth of the vasculature in *Vtn*$^{-/-}$ mice compared to wildtype littermates (Fig. 3B-D).

Another possibility is that since vitronectin is a pericyte gene and pericyte-deficient mice exhibit leaky barriers (*8*, *9*), barrier defects could be due to altered pericyte coverage. However, using NG2:DsRed mice that label mural cells (Fig. 3E), similar pericyte coverage and pericyte density were observed in *Vtn*$^{-/-}$ and wildtype mice (Fig. 3F, G). PDGFRβ protein levels were also similar in *Vtn*$^{-/-}$ and wildtype retinas (Fig. 3H). Hence, vitronectin regulates barrier function without altering pericyte density or pericyte coverage.

Finally, since vitronectin is an ECM protein, we also examined whether the barrier deficits in *Vtn*$^{-/-}$ mice were a consequence of structural and/or functional changes in the ECM. EM analysis of retinal and cerebellum capillaries revealed no obvious structural changes in the basement membrane of endothelial cells in *Vtn*$^{-/-}$ mice compared to wildtype mice. Moreover, immunostaining and western blotting of two highly enriched ECM proteins, collagen IV and fibronectin reveal normal localization (Fig. S4A, B) and expression levels (Fig. S4D) of these proteins in retinas of *Vtn*$^{-/-}$ mice. Notably, we also observed normal collagen IV ensheathment of retinal blood vessels in *Vtn*$^{-/-}$ mice (Fig. S4C). Thus, the overall structural and functional organization of ECM was not compromised in mice lacking vitronectin.

Collectively, our data demonstrate that barrier defects caused in mice lacking vitronectin are not due to defects obvious in vessel morphology and patterning, pericyte coverage or ECM

organization. Rather, pericyte-secreted vitronectin could be acting directly on endothelial cells to regulate their barrier properties.

Vitronectin binding to integrin receptors is essential for barrier function

How does pericyte secreted vitronectin signal to neighboring endothelial cells to regulate barrier permeability? Vitronectin belongs to the family of adhesion proteins that bind integrin receptors through a 3 amino acid motif, Arg – Gly – Asp (RGD) (*21–23*) (Fig. 3I). Previous biochemical studies established that competitive binding experiments using short peptides containing the RGD domain (*24*) as well as mutating vitronectin RGD to RGE (aspartic acid to glutamic acid) effectively abolished binding of vitronectin to integrins (*25*). To determine if vitronectin binding to integrins is essential for barrier function, we took advantage of the knock-in mouse line harboring single point mutation of vitronectin with RGD domain mutated to RGE, $Vtn^{RGE/RGE}$ (*26*), hereafter referred to as Vtn^{RGE}.

Vtn^{RGE} mice exhibited leakage in retina and cerebellum, phenocopying $Vtn^{-/-}$ mice. Similar hotspots of tracer leakage (Fig. 3J) as well as neuronal cell bodies filled with tracer was apparent in the retinal parenchyma of Vtn^{RGE} mice (Fig. 3K, L) with no obvious defects in vascular patterning. The BBB in the cerebellum of these mice was also leaky with tracer hotspots throughout the cerebellum (Fig. S5A, B).

EM analysis of retinas and cerebellum of HRP-injected Vtn^{RGE} mice revealed similar subcellular features in endothelial cells as observed in the $Vtn^{-/-}$ full knockout mice. Both the retina and cerebellum in Vtn^{RGE} mice had functional tight junctions as noted by HRP halting sharply between endothelial cells (Fig. S5C, D). However, we observed >2-fold increase in HRP-filled vesicles in endothelial cells of Vtn^{RGE} mice compared to wildtype animals (Fig. S5E-H), revealing upregulated transcytosis in these mice. Thus, Vtn^{RGE} mice fully recapitulated the phenotype observed in $Vtn^{-/-}$ mice, indicating vitronectin binding to integrin receptors is critical for its role in regulating barrier function.

RGD-binding integrin receptor, α5, is required in endothelial cells for blood-CNS barrier integrity

What are the specific integrin receptors on endothelial cells required for vitronectin-mediated regulation of barrier function? Of the several α and β integrin subunits that heterodimerize to form integrin receptors (*21*) we focused on the two α receptors, α5 and αv that have been shown to interact with RGD-ligands. While αv is considered the predominant receptor for vitronectin, cell motility studies have also demonstrated interaction of vitronectin with α5 (*27*, *28*). Furthermore, α5and αv are known to co-operate during vascular development: they have

overlapping functions and can compensate for the loss of each other (*29*). Finally, single cell RNA-seq data reveals transcripts for both these genes in CNS endothelial cells (*17*).

We first performed in situ hybridization to confirm the localization of these integrin transcripts in the brain. While *Itga5* mRNA co-localized with *Pecam1* (endothelial) mRNA (Fig. 4A), *Itgav* mRNA co-localized with both *Pecam1* as well as *Pdgfrb* (pericyte) mRNA (Fig. S7A). Thus, *Itga5* is predominantly expressed in endothelial cells while *Itgav* is expressed in both pericytes and endothelial cells.

To determine the role of endothelial *Itga5* and *Itgav* in barrier function, we ablated these genes acutely and specifically in endothelial cells by crossing *Itga5*$^{fl/fl}$ or *Itgav*$^{fl/fl}$ (*29*) mice with endothelial cell-specific *Cdh5-CreER* mouse line(*30*). Acute deletion of endothelial *Itga5* (Fig. S6A, B) resulted in a leaky blood-retinal barrier (Fig. 4B) with numerous tracer hotspots spread out in the retina tissue (Fig. 4C, D). Importantly, *Itga5*$^{fl/fl}$*;Cdh5-CreER* mutant mice exhibited normal vascular density and patterning (Fig. S6C-F). Similar to *Vtn*$^{-/-}$ and *Vtn*^{RGE} *mice*, *Itga5*$^{fl/fl}$*;Cdh5-CreER* mutants also had a leaky BBB in the cerebellum (Fig. 4E, F). In contrast to *Itga5*, mice lacking endothelial *Itgav* exhibited normal barrier function. The injected tracer was completely confined to the vasculature in both the retina (Fig. S7B-D) and the brain tissue (Fig. S7E, F) indicating fully sealed blood-CNS barriers in *Itgav*$^{fl/fl}$*;Cdh5-CreER* mice. Collectively, these experiments establish the RGD-specific integrin receptor, α5 as an essential integrin for barrier function, particularly retina and cerebellum. Importantly, mice lacking endothelial Itga5 receptor fully phenocopy *Vtn*$^{-/-}$ and *Vtn*^{RGE} mice. These results demonstrate that ligand-receptor interactions between pericyte-derived vitronectin and endothelial integrin, α5, are critical for regulating barrier properties of CNS endothelial cells *in vivo*.

To directly investigate the role of vitronectin-integrin α5 interactions in vesicular trafficking, we turned to a simplified *in vitro* cell-based assay using mouse brain endothelial cells (bEnd.3) which expresses integrin α5 endogenously (Fig. S8A). When these brain endothelial cells were grown on vitronectin-coated dishes we observed integrin α5 containing adhesive structures (Fig. S8A, B), demonstrating activation of integrin α5 by vitronectin. Furthermore, knockdown of integrin α5 in these cells, resulted in a significant increase in endocytosis (Fig. S8C). In wildtype and scrambled shRNA control cells, we observed very few dye-positive vesicles within the cell. In contrast, in cells depleted of integrin α5, FM1-43 dye was readily incorporated in the newly formed vesicles that were endocytosed from the plasma membrane (Fig. S8C, D). These results together with our *in vivo* data indicate that the engagement of vitronectin-integrin α5 actively inhibits endocytosis in CNS endothelial cells to ensure barrier integrity.

Vitronectin-integrin α5 interaction regulates barrier integrity independent of the caveolae pathway

What is the specific transcytotic pathway downstream of vitronectin-integrin α5 signaling? Caveolae-mediated transcytosis is one pathway that is actively inhibited in CNS endothelial cells by Mfsd2a (*2–4*). To determine whether caveolae-mediated transcytosis is also downstream of vitronectin-integrin signaling, we performed a genetic rescue experiment by crossing *Vtn*$^{-/-}$ mice with *Cav-1*$^{-/-}$ mice (*31*) as caveolin-1 is an obligatory protein required for caveolae formation. To our surprise, genetic ablation of *Cav-1* in *Vtn*$^{-/-}$ mice did not rescue barrier leakage: similar to *Vtn*$^{-/-}$ single knockouts, we observed leakage in *Vtn*$^{-/-}$; *Cav-1*$^{-/-}$ double knockout mice as well (Fig. 4G, H), indicating that vitronectin-mediated inhibition of transcytosis is caveolae-independent. Consistent with this result, we also observed no detectable changes in Mfsd2a localization and expression in the retinas of *Vtn*$^{-/-}$ mice compared to wildtype littermates (Fig. S9A, B). Therefore, vitronectin-integrin α5 mediated regulation of barrier integrity is independent of the caveolae pathway

Discussion

In this study, we demonstrate an essential role for pericyte-derived vitronectin in blood-CNS barrier function. Vitronectin deposited in the extracellular matrix, binds to integrin receptors on CNS endothelial cells and this interaction suppresses transcytosis, thus, regulating barrier integrity. Lack of vitronectin or mutating vitronectin to prevent integrin binding as well as endothelial-specific integrin deletion – all of these genetic manipulations result in dysfunctional blood-CNS barriers, highlighting the role of ligand-receptor interactions of pericyte-to-endothelial signaling in barrier function.

How does pericyte-derived vitronectin interacting with integrin receptors actively inhibit endocytosis in CNS endothelial cells? The adhesive forces generated by the engagement of integrins have been shown in various cell types to maintain plasma membrane tension (*32*, *33*). Disengaging integrin receptors from vitronectin likely causes a reduction in membrane tension and it is well known that decreased membrane tension promotes increased endocytosis (*34–36*). Thus, it is plausible that vitronectin binding to integrin α5 exerts adhesive forces to maintain the plasma membrane tension to ensure low rates of transcytosis in CNS endothelial cells. Our results indicate that signals from perivascular cells to endothelial cells via designated ligand-receptor pairs contribute to the unique biophysical properties of CNS endothelial membrane for barrier integrity.

Our work demonstrates the importance of signaling between perivascular cells and CNS endothelial cells in modulating blood-CNS barriers. It is likely that in addition to vitronectin, other ECM molecules also play key roles in barrier function given that ECM is a hub for several ligand-receptor interactions across multiple cell types. Not surprisingly, ECM breakdown is a hallmark feature associated with many diseases and disorders of the CNS (*37*). Thus, our findings reveal

new molecular targets and pathways within the CNS for development of novel therapeutics that could aid CNS drug delivery.

References

1. T. S. Reese, M. J. Karnovsky, Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**, 207–217 (1967).
2. A. Ben-Zvi, B. Lacoste, E. Kur, B. J. Andreone, Y. Mayshar, H. Yan, C. Gu, Mfsd2a is critical for the formation and function of the blood-brain barrier. *Nature*. **509**, 507–511 (2014).
3. B. J. Andreone, B. W. Chow, A. Tata, B. Lacoste, A. Ben-Zvi, K. Bullock, A. A. Deik, D. D. Ginty, C. B. Clish, C. Gu, Blood-Brain Barrier Permeability Is Regulated by Lipid Transport-Dependent Suppression of Caveolae-Mediated Transcytosis. *Neuron*. **94**, 581-594.e5 (2017).
4. B. W. Chow, C. Gu, Gradual Suppression of Transcytosis Governs Functional Blood-Retinal Barrier Formation. *Neuron*. **93**, 1325-1333.e3 (2017).
5. U. H. Langen, S. Ayloo, C. Gu, Development and cell biology of the blood-brain barrier. *Annu. Rev. Cell Dev. Biol.* **35**, 591–613 (2019).
6. Z. Zhao, A. R. Nelson, C. Betsholtz, B. V. Zlokovic, Establishment and Dysfunction of the Blood-Brain Barrier. *Cell*. **163**, 1064–1078 (2015).
7. P. A. Stewart, M. J. Wiley, Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: A study using quail-chick transplantation chimeras. *Dev. Biol.* **84**, 183–192 (1981).
8. R. Daneman, L. Zhou, A. A. Kebede, B. A. Barres, Pericytes are required for bloodĝ€"brain barrier integrity during embryogenesis. *Nature*. **468**, 562–566 (2010).
9. A. Armulik, G. Genové, M. Mäe, M. H. Nisancioglu, E. Wallgard, C. Niaudet, L. He, J. Norlin, P. Lindblom, K. Strittmatter, B. R. Johansson, C. Betsholtz, Pericytes regulate the blood-brain barrier. *Nature*. **468**, 557–561 (2010).
10. B. P. Heithoff, K. K. George, A. N. Phares, I. A. Zuidhoek, C. Munoz-Ballester, S. Robel, Astrocytes are necessary for blood–brain barrier maintenance in the adult mouse brain. *Glia*. **69**, 436–472 (2021).
11. R. N. Frank, S. Dutta, M. A. Mancini, Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat. *Investig. Ophthalmol. Vis. Sci.* **28**, 1086–1091 (1987).
12. D. Shepro, N. M. L. Morel, Pericyte physiology. *FASEB J.* **7**, 1031–1038 (1993).
13. R. D. Bell, E. A. Winkler, A. P. Sagare, I. Singh, B. LaRue, R. Deane, B. V. Zlokovic, Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. *Neuron*. **68**, 409–427 (2010).
14. D. Y. Park, J. Lee, J. Kim, K. Kim, S. Hong, S. Han, Y. Kubota, H. G. Augustin, L. Ding, J. W. Kim, H. Kim, Y. He, R. H. Adams, G. Y. Koh, Plastic roles of pericytes in the blood-retinal barrier. *Nat. Commun.* **8**, 1–16 (2017).
15. G. A. Strasser, J. S. Kaminker, M. Tessier-Lavigne, Microarray analysis of retinal endothelial tip cells identifies CXCR4 as a mediator of tip cell morphology and branching. *Blood*. **115**, 5102–5110 (2010).
16. L. He, M. Vanlandewijck, E. Raschperger, M. Andaloussi Maë, B. Jung, T. Lebouvier, K. Ando, J. Hofmann, A. Keller, C. Betsholtz, Analysis of the brain mural cell transcriptome. *Sci. Rep.* **6**, 1–13 (2016).
17. M. Vanlandewijck, L. He, M. A. Mäe, J. Andrae, K. Ando, F. Del Gaudio, K. Nahar, T. Lebouvier, B. Laviña, L. Gouveia, Y. Sun, E. Raschperger, M. Räsänen, Y. Zarb, N.

Mochizuki, A. Keller, U. Lendahl, C. Betsholtz, A molecular atlas of cell types and zonation in the brain vasculature. *Nature*. **554**, 475–480 (2018).
18. E. Z. Macosko, A. Basu, R. Satija, J. Nemesh, K. Shekhar, M. Goldman, I. Tirosh, A. R. Bialas, N. Kamitaki, E. M. Martersteck, J. J. Trombetta, D. A. Weitz, J. R. Sanes, A. K. Shalek, A. Regev, S. A. McCarroll, Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell*. **161**, 1202–1214 (2015).
19. G. La Manno, K. Siletti, A. Furlan, D. Gyllborg, E. Vinsland, C. M. Langseth, I. Khven, A. Johnsson, M. Nilsson, P. Lönnerberg, S. Linnarsson, Molecular architecture of the developing mouse brain. *bioRxiv* (2020), doi:10.1101/2020.07.02.184051.
20. H. M. Eilken, R. Diéguez-Hurtado, I. Schmidt, M. Nakayama, H. W. Jeong, H. Arf, S. Adams, N. Ferrara, R. H. Adams, Pericytes regulate VEGF-induced endothelial sprouting through VEGFR1. *Nat. Commun.* **8** (2017), doi:10.1038/s41467-017-01738-3.
21. Richard O. hynes, Integrins: bidirectional, allosteric signaling machines. *Cell*. **1** (2002).
22. K. T. Preissner, Structure and Biological Role of Vitronectin. *Annu. Rev. Cell Biol.* **7**, 275–310 (1991).
23. K. T. Preissner, U. Reuning, Vitronectin in vascular context: Facets of a multitalented matricellular protein. *Semin. Thromb. Hemost.* **37** (2011), pp. 408–424.
24. R. A. Orlando, D. A. Cheresh, Arginine-glycine-aspartic acid binding leading to molecular stabilization between integrin α(v)β3 and its ligand. *J. Biol. Chem.* **266**, 19543–19550 (1991).
25. R. C. Cherny, M. A. Honan, P. Thiagarajan, Site-directed mutagenesis of the arginine-glycine-aspartic acid in vitronectin abolishes cell adhesion. *J. Biol. Chem.* **268**, 9725–9729 (1993).
26. A. K. Wheaton, M. Velikoff, M. Agarwal, T. T. Loo, J. C. Horowitz, T. H. Sisson, K. K. Kim, The vitronectin RGD motif regulates TGF-β-induced alveolar epithelial cell apoptosis. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **310**, L1206–L1217 (2016).
27. J. S. Bauer, C. L. Schreiner, F. G. Giancotti, E. Ruoslahti, R. L. Juliano, Motility of fibronectin receptor-deficient cells on fibronectin and vitronectin: Collaborative interactions among integrins. *J. Cell Biol.* **116**, 477–487 (1992).
28. Z. Zhang, A. O. Morla, K. Vuori, J. S. Bauer, R. L. Juliano, E. Ruoslahti, The αvβ1 integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin. *J. Cell Biol.* **122**, 235–242 (1993).
29. A. Van Der Flier, K. Badu-Nkansah, C. A. Whittaker, D. Crowley, R. T. Bronson, A. Lacy-Hulbert, R. O. Hynes, Endothelial α5 and αv integrins cooperate in remodeling of the vasculature during development. *Development*. **137**, 2439–2449 (2010).
30. Y. Wang, M. Nakayama, M. E. Pitulescu, T. S. Schmidt, M. L. Bochenek, A. Sakakibara, S. Adams, A. Davy, U. Deutsch, U. Lüthi, A. Barberis, L. E. Benjamin, T. Mäkinen, C. D. Nobes, R. H. Adams, Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature*. **465**, 483–486 (2010).
31. B. Razani, J. A. Engelman, X. B. Wang, W. Schubert, X. L. Zhang, C. B. Marks, F. Macalusol, R. G. Russell, M. Li, R. G. Pestell, D. Di Vizio, H. Hou, B. Kneitz, G. Lagaud, G. J. Christ, W. Edelmann, M. P. Lisanti, Caveolin-1 Null Mice Are Viable but Show Evidence of Hyperproliferative and Vascular Abnormalities. *J. Biol. Chem.* **276**, 38121–38138 (2001).
32. A. Katsumi, A. W. Orr, E. Tzima, M. A. Schwartz, Integrins in Mechanotransduction. *J. Biol.*

Chem. **279**, 12001–12004 (2004).
33. T. D. Ross, B. G. Coon, S. Yun, N. Baeyens, K. Tanaka, M. Ouyang, M. A. Schwartz, Integrins in mechanotransduction. *Curr. Opin. Cell Biol.* **25** (2013), pp. 613–618.
34. M. P. Sheetz, J. Dai, Modulation of membrane dynamics and cell motility by membrane tension. *Trends Cell Biol.* **6** (1996), pp. 85–89.
35. A. Diz-Muñoz, D. A. Fletcher, O. D. Weiner, Use the force: Membrane tension as an organizer of cell shape and motility. *Trends Cell Biol.* **23**, 47–53 (2013).
36. J. J. Thottacherry, A. J. Kosmalska, A. Kumar, A. S. Vishen, A. Elosegui-Artola, S. Pradhan, S. Sharma, P. P. Singh, M. C. Guadamillas, N. Chaudhary, R. Vishwakarma, X. Trepat, M. A. del Pozo, R. G. Parton, M. Rao, P. Pullarkat, P. Roca-Cusachs, S. Mayor, Mechanochemical feedback control of dynamin independent endocytosis modulates membrane tension in adherent cells. *Nat. Commun.* **9** (2018), doi:10.1038/s41467-018-06738-5.
37. M. S. Thomsen, L. J. Routhe, T. Moos, The vascular basement membrane in the healthy and pathological brain. *J. Cereb. Blood Flow Metab.* **37** (2017), pp. 3300–3317.
38. T. Yardeni, M. Eckhaus, H. D. Morris, Retro-orbital_injections_in_mice lab Animal 2011.pdf. *Lab Anim*. **40**, 155–160 (2011).

Acknowledgements
The authors thank members of the Gu laboratory for comments on this manuscript; Thomas Sisson for providing the *Vtn*$^{-/-}$ and *Vtn*^{RGE} mice, Ralf Adams for *Cdh5:CreER* mice and David Silver for Mfsd2a antibody.

Funding
This work was supported by Mahoney Postdoctoral Fellowship (S.A.), Fidelity Biosciences Research Initiative (C.G.), an Allen Distinguished Investigator Award (C.G.), R35NS116820 (C.G), NIH DP1NS092473 Pioneer Award (C.G.). The research of C.G. was supported in part by a Faculty Scholar grant from the Howard Hughes Medical Institute. Imaging, consultation and/or services were in part performed in the Neurobiology Imaging Facility. This facility is supported in part by the HMS/BCH Center for Neuroscience Research as part of an NINDS P30 Core Center grant (NS072030). Electron Microscopy imaging, consultation and services were performed in the HMS Electron Microscopy Facility.

Author contributions
S.A. and C.G. conceived the project and designed experiments. S.A., C.G.L. and S.S. performed experiments and analyzed all data. W.Z and B.C designed, performed and analyzed *in vitro* cell culture experiements. S.A. and C.G. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Data and materials availability
Requests for data supporting figures and materials should be addressed to C.G. (chenghua_gu@hms.harvard.edu).

Supplementary materials
Methods
Figures S1-S9

Figure Legends

Fig. 1. Vitronectin expression coincides with functional barrier formation and is enriched in CNS pericytes compared to peripheral tissue pericytes

(A, B) Immunostaining of vitronectin (green) and blood vessels (isolectin, magenta) in retina (A) and (B) brain of P7 mouse.

(C) In situ hybridization for *Vtn* (green), *Pdgfrb* (pericyte gene, magenta) and immunostaining for PECAM1 (vessel marker, red) with DAPI (nuclei, blue) in P7 brain. Dashed lines indicate pericyte nucleus and vessel outline.

(D) In situ hybridization for *Vtn* and *Pdgfrb* in brain and lung of P7 mouse.

(E) Scatter plot between *Pdgfrb* and *Vtn* in brain (green) and lung (red). Each dot represents an individual cell, n=60 and 62 cells respectively across 2 animals. Pearson correlation coefficient $r = 0.82$ for brain and $r = 0.36$ for lung.

Fig. 2. Vitronectin regulates blood-CNS barrier integrity by suppressing transcytosis in CNS endothelial cells

(A) Sulfo-NHS-Biotin (tracer, green) leaks out of blood vessels (isolectin, magenta) in retinas of P10 *Vtn*$^{-/-}$ mice. Arrowheads show tracer hotspots in *Vtn*$^{-/-}$ mice, white boxes correspond to higher magnification images in (B).

(B) Sulfo-NHS-Biotin leaked out in *Vtn*$^{-/-}$ mice taken up by neuronal cell bodies (arrowheads).

(C) Quantification of vessel permeability in retinas of wildtype and *Vtn*$^{-/-}$ mice. n = 5 animals per genotype. Mean ± S.D.; ***$p < 0.001$; Student's t-test.

(D) Leakage of Sulfo-NHS-Biotin (green) from blood vessels (Icam2, magenta) in the cerebellum of P10 *Vtn*$^{-/-}$ mice. White boxes correspond to higher magnification panels shown.

(E) Quantification of vessel permeability in retinas of wildtype and *Vtn*$^{-/-}$ mice. n = 4 animals per genotype. Mean ± S.D.; ***$p < 0.001$; Student's t-test.

(F, G) EM images of HRP halting at tight junctions (arrows) in both retinas (F) and cerebellum (G) of wildtype and *Vtn*$^{-/-}$ mice. Luminal (L) and abluminal sides indicated by dashed yellow lines.

(H, I) EM images showing HRP-filled vesicles (arrowheads) in endothelial cells of retinas (H) and cerebellum (I) of wildtype and *Vtn*$^{-/-}$ mice.

(J, K) Quantification of tracer-filled vesicles in endothelial cells of retinas (J) and cerebellum (K). n = 4 animals per genotype. Mean ± S.D.; ***$p < 0.001$, **$p < 0.01$; Student's t-test.

Fig. 3. Vitronectin is not required for vessel patterning or pericyte coverage and vitronectin binding to integrin receptors is essential for barrier function

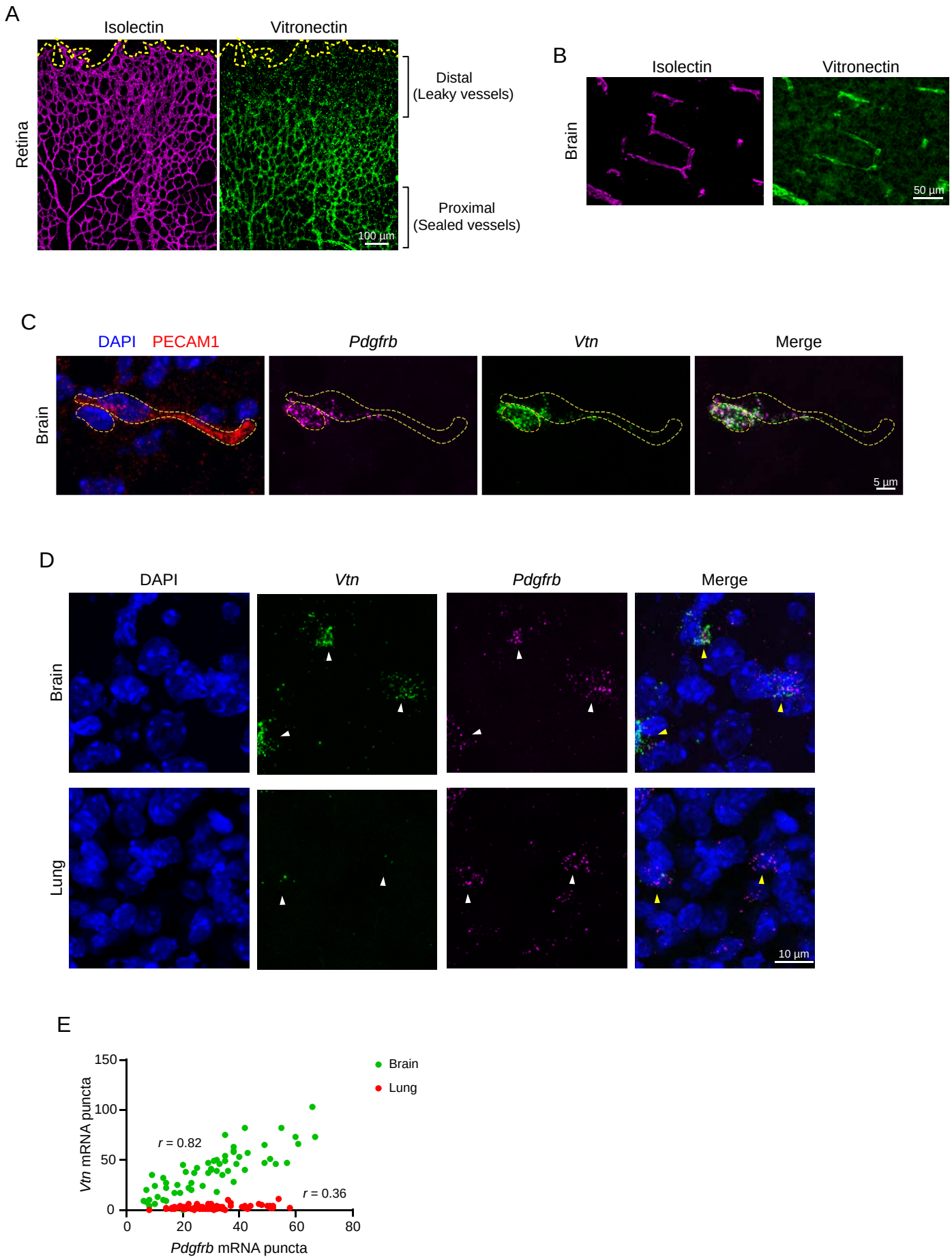
(A) Tilescan images showing retinal vasculature of P10 retinas in wildtype and *Vtn*$^{-/-}$ mice.

(B-D) Quantification of vessel density (B), capillary branching (C) and radial outgrowth (D) in P10 retinas. n = 6 animals per genotype. Mean ± S.D.; n.s. not significant, $p > 0.05$; Student's t-test.

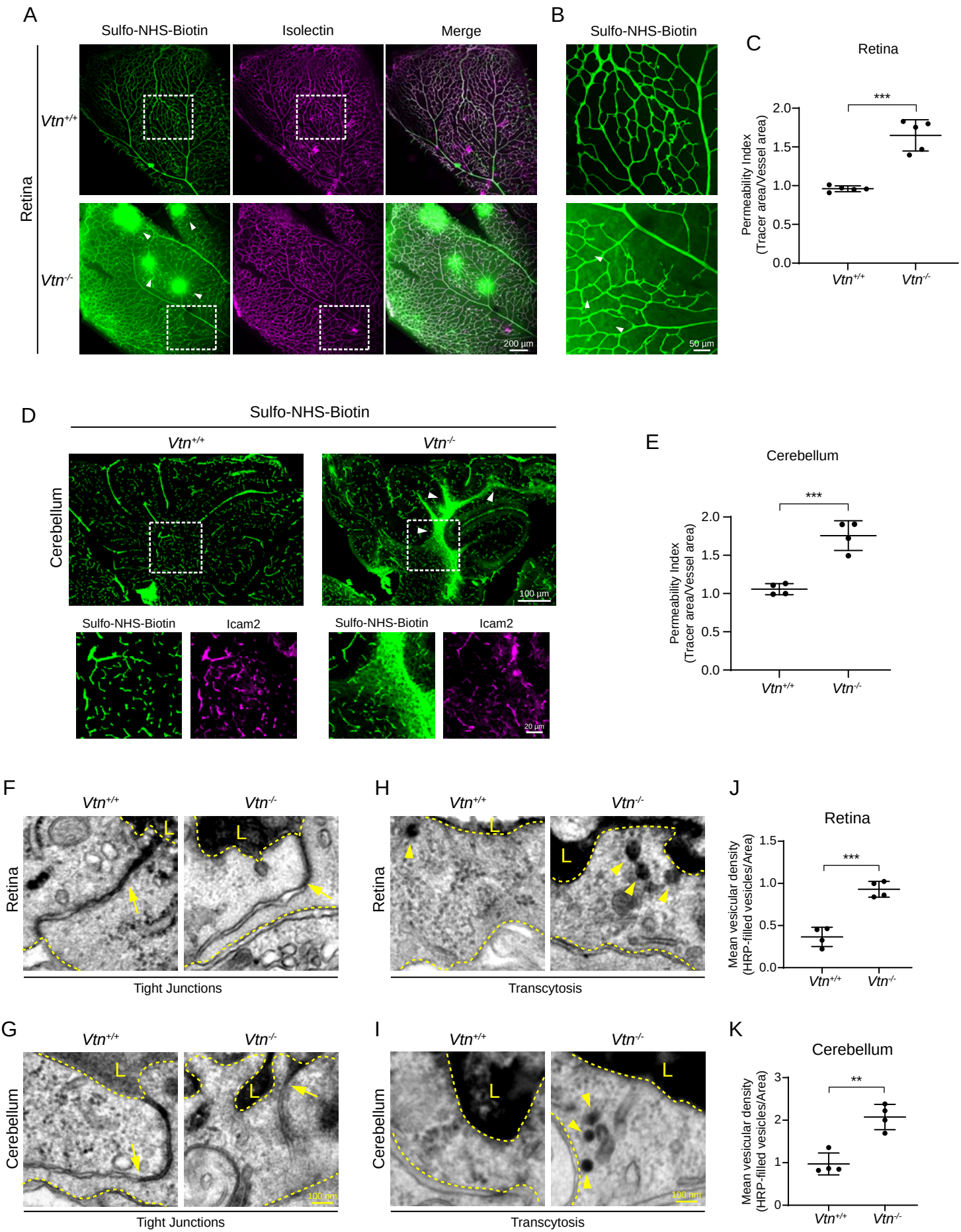
(E) P10 retinas of NG2:DsRed+ wildtype and *Vtn*$^{-/-}$ mice immunostained for ERG1/2/3 (endothelial nuclei, green) and vessels (isolectin, blue).

(F, G) Quantification of pericyte coverage (F) and pericyte density (G) in retinas of wildtype and *Vtn*$^{-/-}$ mice. n = 5 animals per genotype. Mean ± S.D.; n.s. not significant, p > 0.05; Student's t-test.
(H) Representative western blots for PDGFRβ protein levels normalized to Gapdh in whole retinal lysates of P10 wildtype and *Vtn*$^{-/-}$ mice.
(I) Schematic illustrating binding of ligands containing RGD-motif with integrin receptors
(J) Leakage of Sulfo-NHS-Biotin (tracer, green) from vessels (isolectin, magenta) in P10 *Vtn*^{RGE} mice. White boxes correspond to higher magnification images shown in (K).
(K) Tracer hotspots (arrowheads) in retinas of *Vtn*^{RGE} mice.
(L) Quantification of vessel permeability in wildtype and *Vtn*^{RGE} mice. n = 5 animals per genotype. Mean ± S.D.; ***p < 0.001; Student's t-test.

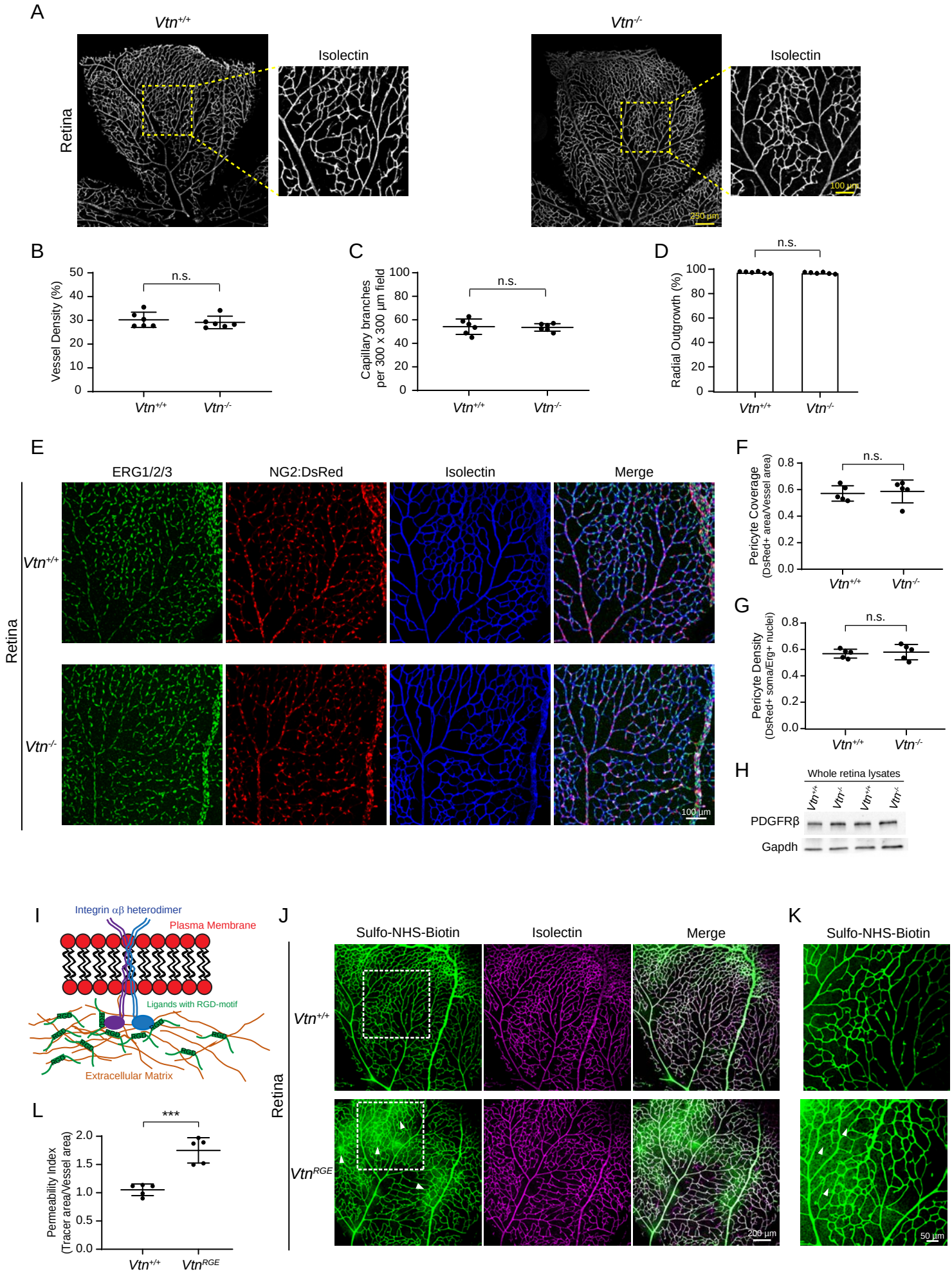
Fig. 4. *Itga5* in endothelial cells is specifically required for blood-CNS barrier function and vitronectin-integrin α5 mediated barrier regulation is independent of the caveolae pathway
(A) In situ hybridization for *Itga5* (green), *Pecam1* (endothelial gene, red) and *Pdgfrb* (pericyte gene, magenta) in P7 brain tissue.
(B) Sulfo-NHS-Biotin (tracer, green) leaks out of vessels (isolectin, magenta) in P10 *Cdh5:CreER+; Itga5*$^{fl/fl}$ mice. Tamoxifen administered P3-P5, see Figure S6. White boxes correspond to higher magnification images in (C).
(C) Tracer hotspots (arrowheads) in mice lacking endothelial Itga5.
(D) Quantification of vessel permeability in wildtype and *Cdh5:CreER+; Itga5*$^{fl/fl}$ mice. n = 5 animals per genotype. Mean ± S.D.; **p < 0.01; Student's t-test.
(E) Sulfo-NHS-Biotin (green) leakage in cerebellum of P10 *Cdh5:CreER+; Itga5*$^{fl/fl}$ mice. White boxes correspond to higher magnification images with tracer and vessels (ICAM2, magenta).
(F) Quantification of vessel permeability in wildtype and *Cdh5:CreER+; Itga5*$^{fl/fl}$ mice. n = 5 animals per genotype. Mean ± S.D.; ***p < 0.001; Student's t-test.
(G) Deletion of caveolin-1 does not rescue tracer leakage seen in *Vtn*$^{-/-}$ mice. Sulfo-NHS-biotin leakage is observed in *Vtn*$^{-/-}$*; Cav-1*$^{-/-}$ double knockouts, similar to *Vtn*$^{-/-}$ single knockout mice.
(H) Quantification of vessel permeability in P10 retinas across all genotypes. n = 4 animals per genotype. Mean ± S.D.; ***p < 0.001, n.s. not significant, p > 0.05; one-way ANOVA with Tukey's post hoc test.



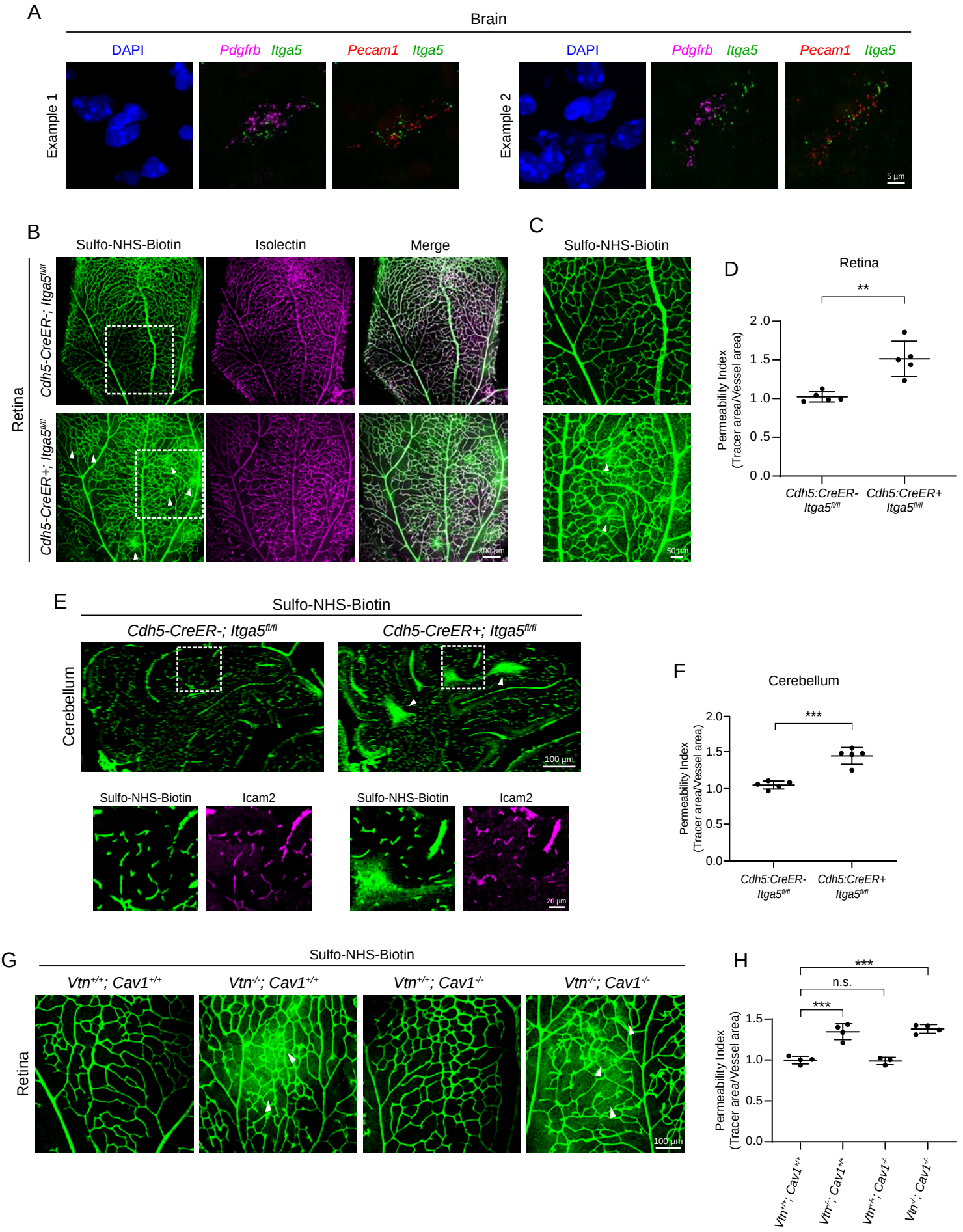
Ayloo et al. Figure 1



Ayloo et al. Figure 2



Ayloo et al. Supplementary Figure 3



Ayloo et al. Figure 4