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<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/pnas.1415316111">http://dx.doi.org/10.1073/pnas.1415316111</a></td>
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<tr>
<td>Publisher</td>
<td>National Academy of Sciences (U.S.)</td>
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<tr>
<td>Version</td>
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Constitutively active Notch4 receptor elicits brain arteriovenous malformations through enlargement of capillary-like vessels

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Edited by Michael A. Gimbrone, Brigham and Women’s Hospital, Boston, MA, and approved November 11, 2014 (received for review August 13, 2014)

Arteriovenous (AV) malformation (AVM) is a devastating condition characterized by focal lesions of enlarged, tangled vessels that shunt blood from arteries directly to veins. AVMs can form anywhere in the body and can cause debilitating ischemia and life-threatening hemorrhagic stroke. The mechanisms that underlie AVM formation remain poorly understood. Here, we examined the cellular and hemodynamic changes at the earliest stages of brain AVM formation by time-lapse two-photon imaging through cranial windows of mice expressing constitutively active Notch4 (Notch4*). AVMs arose from enlargement of preexisting microvessels with capillary diameter and blood flow and no smooth muscle cell coverage. AV shunting began promptly after Notch4* expression in endothelial cells (ECs), accompanied by increased individual EC areas, rather than increased EC number or proliferation. Alterations in Notch signaling in ECs of all vessels, but not arteries alone, affected AVM formation, suggesting that Notch functions in the microvasculature and/or veins to induce AVM. Increased Notch signaling interfered with the normal biological control of hemodynamics, permitting a positive feedback loop of increasing blood flow and vessel diameter and driving focal AVM growth from AV connections with higher blood velocity at the expense of adjacent AV connections with lower velocity. Endothelial expression of constitutively active Notch1 also led to brain AVMs in mice. Our data shed light on cellular and hemodynamic mechanisms underlying AVM pathogenesis elicited by increased Notch signaling in the endothelium.


Significance

Brain arteriovenous malformations are focal lesions of enlarged, tangled vessels that shunt blood from arteries directly to veins. They can cause ischemia, hemorrhage, disability, and death, particularly in young people, accounting for 50% of childhood stroke. The molecular etiology of the disease remains poorly understood, hindering the development of therapeutic treatments. Here, we report that, in an animal model, the lesion arises from the enlargement of capillary-like vessels. Notch signaling in the endothelium of microvasculature and veins is critical for the disease initiation by increasing cell areas but not proliferation. Blood flow mediates disease progression by a positive feedback of increasing flow and vessel diameter. Our data shed light on the mechanism underlying the pathogenesis of this devastating disease.

PNAS | December 16, 2014 | vol. 111 | no. 50 | 18007–18012

www.pnas.org/cgi/doi/10.1073/pnas.1415316111

MEDICAL SCIENCES
ECs results in spontaneous AVMs in mice (15–17, 21). Although this work has facilitated new research directions regarding the Notch pathway in AVMs, how aberrant Notch signaling leads to AVM remains unknown. Elucidating the initiating structural events that lead to AV shunt formation would provide insight into AVM pathogenesis.

A major obstacle to understanding AVM pathogenesis has been the inability to observe AVM formation and blood flow over time with high-resolution in vivo imaging. Here, we examined AVM formation in Tie2-Notch4* mice from the initial genetic event, illuminating disease progression using “5D” two-photon imaging, which allows high-resolution live imaging of vascular architecture (3D) and blood velocity (the fourth dimension) over time (the fifth dimension) (16, 22, 23). Our findings provide insights into the mechanism of brain AVM formation.

Results

Notch4*-Mediated AV Shunts Arise from the Enlargement of Capillary-Like Vessels. To determine the developmental origin of AVMs, we performed longitudinal live imaging in the brains of Tie2-Notch4* mice, in which Notch4* expression was repressed until birth by tetracycline treatment. We implanted cranial windows over the right parietal cortex of mice at postnatal day 7 (P7) and used in vivo two-photon microscopy to record vessel diameter and blood velocity over time. We measured lumen diameters of vessels connecting arteries and veins, or AV connections, at their narrowest point. We defined measurements ≥12.5 μm as AV shunts because AV connections of this diameter were not observed in control mice after P12 (SI Appendix, Fig. S1). We found that AV shunts developed from capillary-like vessels in Tie2-Notch4* mice beginning at ~P12 (Fig. 1 and SI Appendix, Figs. S2 and S3). Of 109 of the AV connections that we tracked, 41 (38%) grew to ≥12.5 μm between P14 and P25 in 12 mutants, and of 109 (4%) grew to ≥30 μm (Fig. 1 and Movies S1 and S2). However, the initial diameter did not differ from that of controls (5.4 ± 1.6 vs. 5.3 ± 1.6 μm, Fig. 1B). We saw no correlation between the final and initial diameters of AV shunts (SI Appendix, Fig. S4). We conclude that AV shunts in Tie2-Notch4* mice enlarge from capillary-diameter AV connections, but that only a few continue to grow into large AVMs.

We examined the hemodynamics of AV shunts at their onset and as they enlarged. Using 5D two-photon imaging (16, 23), we simultaneously analyzed red blood cell velocity and AV connection diameter (Fig. 1 C–E and SI Appendix, Fig. S2). AV...
connection enlargement either coincided with or preceded the initial increase in velocity (Fig. 1C, 8/18 vessels in four mice). Some capillary-like vessels enlarged without a significant increase in velocity (Fig. 1C, 4/18 vessels in four mice), whereas others exhibited an increase in neither diameter nor velocity (Fig. 1C, 6/18 vessels in four mice). Neither velocity nor diameter increased in controls (Fig. 1D, 18 vessels in four mice). Data are summarized in Fig. 1E. Thus, our data suggest that blood velocity in AV connections that grew into AV shunts is initially capillary-like and becomes abnormally elevated only when connections enlarge.

As capillaries lack smooth muscle cell (SMC) coverage, we performed staining for α-smooth muscle actin (SMA) at P12, when AV shunts were first apparent. We identified 10 AV shunts from five mice that lacked SMA staining (Fig. 1F). In contrast, adjacent arteries were positive for SMA staining, similar to controls (Fig. 1G and SI Appendix, Fig. S5). We observed, as we have previously published (15–17), that advanced AVMs exhibited increased SMA staining. Thus, the absence of SMCs in the initial AV shunts is consistent with a capillary origin of AVMs in the Tie2-Notch4* mice.

We next examined the expression of Notch4* during the initiation of AV shunting. We previously demonstrated that Tie2-tTA effectively and specifically drives gene expression throughout the brain endothelium, through activation of the tetracycline response element (TRE) by the tetracycline transactivator (TfA) (17). Here we performed whole-mount staining of brain vasculature for the Notch4 intracellular domain (ICD), which also detects Notch4*, without the extracellular domain. We detected elevated Notch4-ICD throughout vasculature (reflecting Notch4* expression) in the mutants over the controls (SI Appendix, Fig. S6).

To examine the kinetics of Notch4* expression in real time during AV shunt formation, we analyzed TRE-H2b-eGFP, Tie2-tTA; TRE-Notch4* mice. TRE-H2b-eGFP reports TfA activity and thus TRE-Notch4* expression. As expected, there was a positive correlation between H2b-eGFP intensity and Notch4* expression in fixed samples, validating the reporter assay (SI Appendix, Fig. S7). We found that TfA activity preceded, by about 2 d, the enlargement in each segment (branch point to branch point) of the AV connections measured (Fig. 2A–C). Approximately 80% (159/198) of the segments were positive for the TRE-H2b-eGFP reporter (Fig. 2D and SI Appendix, Fig. S8). About 50% (83/159) of the positive segments enlarged, and 40% (59/159) of these became part of AV shunts ≥12.5 μm in diameter. Only 15% (24/159) regressed (Fig. 2D and SI Appendix, Fig. S8). In contrast, only 10% of the TRE-H2b-eGFP-negative segments enlarged (4/39); 80% (31/39) regressed. These data suggest that Notch4* expression promptly leads to AV shunting.

EC Area, but Not Number or Proliferation, Increased in AV Shunt Formation. To examine the cellular changes underlying the initiation of AV shunts, we used the R26R-confetti reporter (24) to track the position and number of labeled cells. We activated the mT/mG Cre-reporter throughout the vascular endothelium, through activation of the tetracycline responsive element (TRE) by the tetracycline transactivator (TfA) (17). Here we performed whole-mount staining of brain vasculature for the Notch4 intracellular domain (ICD), which also detects Notch4*, without the extracellular domain. We detected elevated Notch4-ICD throughout vasculature (reflecting Notch4* expression) in the mutants over the controls (SI Appendix, Fig. S6).

We hypothesized that a change in the area covered by individual ECs might support the capillary enlargement in Tie2-Notch4* mice. To test this, we stained whole-mount cortical sections of mutants and controls for vascular endothelial cadherin (VE-cadherin, Cdh5) during the initial stages of AV shunt formation (P12). We found that the area covered by individual ECs increased more than threefold during the early stages of AV shunt formation (Fig. 3E and F). Mean cell area of 157 ± 24 μm² in three Tie2-tTA mice and 540 ± 46 μm² in five Tie2-tTA;TRE-Notch4* mice with AV shunts ≥12.5 μm; P = 0.0001 by two-tailed Student t test). Thus, an increase in EC area correlates with the enlargement of capillaries in AV shunt formation.

Notch4* Expression in ECs of Arteries Was Not Sufficient to Induce AV Shunt Formation. We asked where in the vascular tree Notch4* acts to elicit AV shunt formation. Our in vivo imaging suggested that a defect in the capillaries or microcirculation led to AV shunts. Because no capillary EC-specific tTA driver has been reported, we used Cdh5(PAC)-CreERT2 to induce Notch4* in all ECs and a recently developed arterial-specific line, BMX(PAC)-CreERT2, to examine the effects of Notch4* specifically in the arterial ECs.

We first confirmed the expression of the two inducible Cre lines in our system. Cdh5(PAC)-CreERT2 efficiently activated a mTmG Cre-reporter throughout the vascular endothelium, but not in circulating blood cells (SI Appendix, Figs. S11 and S12). The ROSA:LNL-tTA system effectively induced Notch4* expression, resulting in even higher protein levels than Tie2-tTA-driven expression (SI Appendix, Fig. S13 and Movie S3) (25). As demonstrated in other tissues (26), BMX(PAC)-CreERT2 efficiently activated the same reporter throughout the arterial trunk.
and cells. ECs in cerebrovascular arterioles showed positive arteriovenous connections.

Changes in Notch signaling in the artery alone did not affect AV shunt formation. To investigate the molecular mediator of Notch4*-induced AV shunt formation, we examined Rbpj, a transcription factor required for canonical Notch signaling. We deleted Rbpj in Tie2-tTA; TRE-Notch4*; Cdh5(PAC)-CreERT2; Rbpj<sup>fl/fl</sup> mice before the onset of AV shunts by injecting TAM at P7–P8. Excision of Rbpj using Cdh5(PAC)-CreERT2 effectively suppressed AV shunt formation, and only 1.2 ± 1.7% of total AV connections were enlarged by P18 (Fig. 4D and SI Appendix, Table S2). In contrast, in controls with one remaining Rbpj allele, 22.3 ± 17.2% of total AV connections were enlarged (Fig. 4E and SI Appendix, Table S2). Thus, Notch4*-induced AV shunts require canonical Notch signaling via Rbpj.

Because AV shunts were Rbpj-dependent, we tested whether blocking arterial Notch signaling suppresses AV shunt formation. We deleted Rbpj by injecting TAM at P7–P8 in arterial ECs of Tie2-tTA; TRE-Notch4*; BMX(PAC)-CreERT2; Rbpj<sup>fl/fl</sup> mice. Excision of both Rbpj alleles using BMX(PAC)-CreERT2 did not suppress the AV shunt phenotype, because we found that 62.7 ± 27.5% of total AV connections were enlarged by P18 (Fig. 4F and SI Appendix, Table S2), which is different from mice with excision of only one Rbpj allele [60.1 ± 36.4% of total AV connections enlarged (SI Appendix, Table S2)]. Taken together, these data suggest that the onset of AV shunts is mediated through Rbpj in the ECs of microvasculature and veins but not BMX(PAC)-CreERT2–positive arteriovenous connections were enlarged. By contrast, the BMX-CreERT2; ROSA:LNL:tTA; TRE-Notch4<sup>+</sup> mice resembled the negative littermate controls with only 2.1 ± 3.6% of total AV connections enlarged (Fig. 4B and C and SI Appendix, Table S1). Therefore, expression of Notch4* in the endothelium of BMX-CreERT2 positive arteries is not sufficient to induce AV shunts in the brain.

Rbpj in the ECs of BMX-CreERT2–negative, but Not BMX-CreERT2–Positive Arteries, Was Required for Notch4*-Induced AV Shunt Formation. To investigate the molecular mediator of Notch4*-induced AV shunt formation, we examined Rbpj, a transcription factor required for canonical Notch signaling. We deleted Rbpj in Tie2-tTA; TRE-Notch4*; Cdh5(PAC)-CreERT2; Rbpj<sup>fl/fl</sup> mice before the onset of AV shunts by injecting TAM at P7–P8. Excision of Rbpj using Cdh5(PAC)-CreERT2 effectively suppressed AV shunt formation, and only 1.2 ± 1.7% of total AV connections were enlarged by P18 (Fig. 4D and SI Appendix, Table S2). In contrast, in controls with one remaining Rbpj allele, 22.3 ± 17.2% of total AV connections were enlarged (Fig. 4E and SI Appendix, Table S2). Thus, Notch4*-induced AV shunts require canonical Notch signaling via Rbpj.

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investigated whether differences in blood flow velocity determine the growth of AV shunts. We identified arterial branch points in mutant mice in which one branch clearly led into an AV shunt (V1 in Fig. 5 and B). We measured velocity and diameter in the higher-velocity AV shunt and the lower-velocity adjacent artery (V2 in Fig. 5 A and B) over time in Tie2-Notch4* mice and compared the changes in velocity and diameter to control arteries (V1 and V2 in Fig. 5 C and D). The velocities in control arteries were more stable, varying by an average of 16% between time points vs. 45% in the mutants. In five of seven cases where the velocity in the AV shunt increased, the velocity in the adjacent artery with lower velocity decreased (compare Fig. 5 A to B; see summary in Fig. 5E “when AV shunt velocity increases” (see example in Movie S4)). In three cases, the velocity was reduced in both the AV shunt and its adjacent artery (Fig. 5E, “when AV shunt velocity decreases”). The velocity in control arteries did not change (Fig. 5E, “control”). Thus, increased velocity in AV shunts is often accompanied by decreased velocity in the adjacent arteries of Tie2-Notch4* mice.

We then investigated whether changes in velocity correlated with changes in diameter in Tie2-Notch4* mutants. We focused on the AV shunts in which velocity increased and adjacent arteries in which velocity decreased (represented by red dots in Fig. 5E). We found that the adjacent downstream arteries indeed regressed, compared with either the AV shunt or arteries measured in control mice (Fig. 5F, P < 0.02 by two-tailed Student t test, V2 in Fig. 5 A and B, and SI Appendix, Fig. S15). Therefore, changes in velocity correlated with changes in diameter, so that the highest velocity connections, which also tended to be the most proximal AV connections in the Tie2-Notch4* mice, exhibited increasing velocity and diameter, whereas the lower velocity and typically distal arterial branches exhibited reduced velocity and diameter.

For a broader perspective on AV shunt progression, we examined the whole brain by casting the vessels with radio-opaque microfil and imaging with microCT. Despite widespread AV shunting in mutant mice, AVM development appeared focal (Fig. 5G). Thus, our data suggest that the unchecked growth of higher-velocity AV connections results in focal AVM development.

**Endothelial Expression of Activated Notch1 Induced AV Shunt Formation.** To determine whether expression of constitutively active Notch1 in the endothelium also causes AV shunt formation, we combined a constitutively active, tTA-responsive Notch1* allele (16) with Tie2-tTA and activated Notch1* at birth. Five of five Tie2-tTA;TRE-Notch1* mice exhibited signs of ataxia or lethargy by P14, as well as large brain AVMs, whereas none of six controls were affected (Fig. 6). Thus, endothelial expression of constitutively active Notch1 induces brain AV shunts during neonatal development.

**Discussion**

Here, we provide novel mechanistic insights into AVM formation. Our data suggest that AV shunts arise from the enlargement of capillary-like vessels and that cell-autonomous Notch signaling in the endothelium of microvasculature and veins is sufficient and required for AVM formation. AV shunt initiation is accompanied by the enlargement of EC area but not increased proliferation. Blood flow mediates AV shunt progression by positive feedback of increasing flow and increasing diameter, leading to the selective growth of focal AVMs.

Our data shed light on the origin of AVMs. The vascular structure of brain AVMs has led to speculations that they arise from failed regression of primitive AV connections during development (3, 4). Extrapolation of histological analysis of skin AVMs led others to propose that brain AVMs arise from the dilatation of postcapillary venules (5). In contrast to the existing models, our results demonstrate that Notch4*-induced AV shunts grow from preexisting capillary-like connections with no detectable differences from normal capillaries in either their diameter or their velocity. This suggests a new, potentially capillary-originated model of AVM initiation.

Endothelial but not hematopoietic Notch signaling is critical in the formation of AV shunts. Previous work has demonstrated a critical function for blood cells in separating the blood and lymphatic vascular systems (27). Our data, confirmed by two independent induction systems, demonstrate that increased Notch signaling specifically within the endothelium is sufficient to cause AV shunt formation without obvious involvement of the blood cells. Conversely, expression of Notch4* specifically in Sch/tTA–positive blood lineages did not lead to AV shunt formation (SI Appendix, Fig. S16). Furthermore, no increase in CD45+ cells was observed near AV shunts (SI Appendix, Fig. S17). Together, our data suggest that expression of Notch4* in the blood lineages is insufficient to induce AV shunts and that endothelial Notch4* elicits AVMs in a cell-autonomous fashion.

Initial enlargement of AV shunts correlates with area expansion of individual ECs, rather than increased proliferation. Consistent with this finding, we found that the converse is true: EC area is reduced during AV shunt regression upon turning off the
Notch4* transgene (16). Further investigation is required to determine whether an altered cell area is a direct or indirect effect of Notch4*. Whether further enlargement of AV shunts beyond the initial stage depends on cell proliferation is currently unknown. We speculate that the growth of the AV shunts eventually requires cell proliferation because AV shunts in symptomatic mice can be >10-fold larger than normal capillaries.

Notch signaling in the microvasculature and veins is both sufficient and required for AVM formation. Arterial BMX(PAC)-CreERT2-mediated up- or down-regulation of Notch signaling could not induce or suppress AV shunt formation, respectively. In the same genetic systems, global endothelial Cdh5(PAC)-CreERT2-mediated up- or down-regulation could. These findings are consistent with recent work showing that Rbpj-mediated arterial Notch signaling is dispensable for postnatal artery formation in the retina (26). Together, these data show that changes in Notch signaling in the BMX(PAC)-CreERT2 active cells are not critical in AV shunt formation, implying that it induces AVM through effects on the microvasculature and veins.

Blood flow is a key regulator of vessel caliber (28). Based on clinical studies, it has been proposed that increased flow through low-resistance AV shunts encourages their growth while “stealing” blood flow from adjacent higher-resistance vessels (29). How such vessels evasively grow is focused on a single time point with lower resolution than we show here. We provide experimental evidence that endothelial Notch4* permits a “steal” and perpetuates a positive feedback loop, leading to selective growth of higher-velocity connections at the expense of lower-velocity connections.

Our data also demonstrate that, in controls, blood velocity in adjacent two-branch arteries is maintained at a comparable rate over time. In mutants, the blood velocity in adjacent two-branch arteries varies greatly and with increasing disparity. Our earlier work shows that turning off Notch4* expression normalizes high-velocity AV shunts by rapidly reducing the flow and diameter of the AV shunt while increasing flow and diameter to the distal artery (16). Together, these findings suggest that sustained endotheal expression of Notch4* compromises biological checks that prevent AV connections from enlarging and “stealing” flow from adjacent vessels.

We believe that normal AV specification is critical in maintaining proper hemodynamics in the vasculature. Because Notch signaling is a critical determinant of AV specification, it is conceivable that either gain or loss of Notch activity could disrupt AV specification, compromising normal vascular control of hemodynamic forces and leading to AVMs. Indeed, both gain- and loss-of-function Notch mutants develop abnormal shunting in mouse embryos (13, 30). Our model is consistent with recent work hinting that reduced Notch signaling in HHT type 2 could contribute to AVMs (31). We propose that increased Notch signaling in the endothelium of nonarterial vessels disturbs bidirectional control of blood flow by allowing the persistent growth of high-flow AV connections at the expense of lower flow AV connections (SI Appendix, Fig. S18).

Materials and Methods
See SI Appendix for detailed information on materials and methods. Cranial windows, in vivo imaging, lectin-perfusion, Notch4-ICD, SMA, and VE-cadherin immunostaining was as described (16). BrdU immunostaining was as described (22). For peripheral blood isolation, blood was removed from right ventricles and erythrocytes were lysed. Flow cytometry was performed following CD45 immunostaining and DAPI labeling. For MicroCT, images of Microfil-labeled vasculature were acquired using a μCT 40 system (Scanco Medical AG). All mouse lines used have been published. This study was carried out in strict accordance with National Institute of Health regulations and the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Acknowledgments
This work was supported by National Institutes of Health (NIH) Grants R01 NS067420 and R55NS057472, Vascular Cures (formerly the Pacific Vascular Research Foundation), the Frank A. Campini Foundation, the Mildred V. Strous Trust, American Heart Association (AHA) Grant-in-Aid 10R8NT1471046 and GRNT 16850032, NIH Grant R01 HL075033 (to R.A.W.), AHA Grant 0715002Y and Tobacco-Related Disease Research Program (TRDRP) 18DT-0009 Predoctoral Fellowships (to P.A.M.), TRDRP TNT-007 and NIH F30 1F30HL099005-01A1 Predoctoral Fellowships (to T.N.K.), and TRDRP 20FT-0069 and NIH F32 1F32HL110724-01A1 Postdoctoral Fellowships (to C.M.N.).