Chloride intracellular channel 4 is required for maturation of the cerebral collateral circulation

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Lucitti JL, Tarte NJ, Faber JE. Chloride intracellular channel 4 is required for maturation of the cerebral collateral circulation. Am J Physiol Heart Circ Physiol 309: H1141–H1150, 2015. First published August 14, 2015; doi:10.1152/ajpheart.00451.2015.—The number and diameter of native collaterals in tissues of healthy mice vary widely, resulting in large differences in tissue injury in occlusive diseases. Recent studies suggest similar variation may exist in humans. Collateral variation in mice is determined by genetic background-dependent differences in embryonic collateral formation, by variation in maturation of the nascent collaterals, and by environmental factors such as aging that cause collateral rarefaction in the adult. Recently, formation of the collateral circulation in the brain was found to involve a unique VEGF-A-dependent “arteriolar” angiogenic sprouting-like mechanism. Elsewhere, chloride intracellular protein 4 (CLIC4) was implicated but not investigated directly, prompting the present study. Deletion of Clic4 had no effect on embryonic collaterogenesis. However, during collateral maturation from embryonic day 18.5 to postnatal day 7, reduced mural cell investment was observed and excessive pruning of collaterals occurred. Growth in collateral diameter was reduced. This resulted in 50% fewer collaterals of smaller diameter in the adult and thus larger infarct volume after middle cerebral artery occlusion. During collateral maturation, CLIC4 deficiency resulted in reduced expression of Vegfr2, Vegfr1, Vegfc, and mural cell markers, but not notch-pathway genes. Overexpression of VEGF-A in Clic4−/− mice had no effect on collaterogenesis, but rescued the above defects in collateral maturation by preventing mural cell loss and collateral pruning, thus restoring collateral number and diameter and reducing stroke severity in the adult. CLIC4 is not required for collaterogenesis but is essential for perinatal maturation of nascent collaterals through a mechanism that supports VEGF signaling.

collateral circulation; stroke; CLIC4; VEGF; embryo

NEW & NOTEWORTHY

Chloride intracellular channel-4 (CLIC4) is not required for embryonic collateral arteriole formation in the brain but is required for perinatal collateral maturation. Vegfa overexpression in CLIC4-deficient mice partially rescues deficits in perinatal collateral mural cell investment, and fully rescues aberrant perinatal collateral pruning and enlarged infarct volume after stroke in adults.

Cardiovascular disease and stroke are leading causes of morbidity and mortality in developed countries, and together claim annually ~800,000 lives in the US (28). Native (preexisting) collaterals in tissues limit the severity of ischemic tissue damage (24, 34, 35). These arteriole-to-arteriole anastomoses connect adjacent arterial trees and provide alternative routes for perfusion if the trunk or major branch of one of the trees becomes obstructed. In the cerebral cortex, collaterals reside within the pia mater and interconnect the middle, anterior, and posterior cerebral artery (MCA, ACA, PCA) trees. The number and diameter (“extent”) of native collaterals in brain and other tissues varies widely among different inbred strains of healthy adult mice due to genetic polymorphisms, resulting in large differences in tissue injury in models of stroke and ischemic disease (33). Collateral-dependent perfusion also varies widely among humans with similar impact, although the sources of this variation remain unclear (24, 34, 35).

Despite their importance, relatively little is known about how native collaterals form. By embryonic (E) day 12.5 in mice, the nascent MCA tree has begun to form from remodelling of the cerebral plexus (5). Pial collaterals subsequently begin to appear between the distal branches of the MCA and ACA trees as the latter’s branches emerge from the central sulcus at approximately E14.5 (21). At this time, the trunk and major branches of the MCA tree are well-formed and most of the distal-most arterioles in its crown turn and penetrate into the parenchyma. A small fraction of these arterioles form angiogenic-like sprouts just before turning downward. These sprouts migrate across the underlying pial capillary plexus as an endothelial cell (EC) cord and fuse with a distal arteriole of the opposing arterial tree, followed by lumen formation within the cord (21). This process of collaterogenesis ends near E18.5 (5, 21). Although the molecular mechanisms underlying it are undoubtedly complex, details are emerging: Collaterogenesis involves paracrine vascular endothelial growth factor-A (VEGF-A) signaling via VEGFR2 and the VEGFR2-Notch signaling pathway (21). Knockdown of non-endothelial Vegfa, global Flk1, Adam10, or inhibition of gamma-secretase activity prior to collaterogenesis reduce collateral formation without affecting overall cerebral vascular architecture. Furthermore, these embryos never recover collateral number, resulting in deficiency in adulthood and worse stroke.

The number of collaterals that form during collaterogenesis is the major determinant of the number present in the adult. However, a second process, postnatal maturation, also contributes. Maturation extends from E18.5 through approximately postnatal day (P) 21, depending on mouse strain, and consists of a pruning away of a percentage of the nascent collaterals (5, 10). Those that remain undergo wall maturation, increase their diameter and length, and begin to acquire their characteristic tortuosity.

Chloride intracellular channel-4 (CLIC4) is a member of a 7-membrane-spanning family of proteins (CLICs), and is one of two members expressed in ECs and other vascular wall cells, the other being CLIC1 which when deleted has no effect on collaterogenesis or collateral maturation (8). Usually resid-
ing in the cytoplasm, CLIC4 can also be found in the mitochondria, endoplasmic reticulum, nucleus and plasma membrane, and translocates between compartments (3, 22, 31, 37, 39), including to the nucleus during metabolic stress, growth arrest, apoptosis, and TGFβ signaling (40). Several studies have begun to define vascular functions of CLIC4. Knockdown of CLIC4 in vitro impairs EC proliferation and formation of EC cords, intracellular vacuoles, and the tubular plexus (42, 43). In vivo, CLIC4 deletion disrupts matrigel and ischemia-induced neoangiogenesis (3, 42, 43). We previously uncovered a possible role for CLIC4 in collaterogenesis and showed that deficiency causes reduction of collateral extent in the adult brain and hindlimb resulting in worse ischemic injury, while not affecting the general arterial-venous circulation (6).

The purpose of the present study was to determine the role of CLIC4 in cerebral collaterogenesis. Deletion of CLIC4 had no effect on embryonic collateral formation. However, deficiency impaired mural cell recruitment and caused extensive collateral pruning between E18.5 and P7, which resulted in reduced number and smaller diameter of collaterals in the adult and thus more severe strokes. Overexpression of Vegfa in Clic4−/− mice completely prevented these defects and partially restored P4 collateral mural cell coverage. Isolated pial membranes from P4 and P7 Clic4−/− mice had reduced expression of Vegfa pathway and mural cell genes. These findings provide a link between CLIC4 and VEGF signaling in vivo and establish that CLIC4 is required for perinatal pial mural cell investment and collateral maturation.

MATERIALS AND METHODS

Animals

Clic4−/− mice originally generated on a CD1 background were backcrossed at least 8 generations to C57BL/6 (Charles River Laboratories no. 027). After further outcrossing to C57BL/6J (Jackson Laboratory no. 00664), heterozygous F1 progeny were intercrossed to produce related wild-type (WT) (Clic4+/+) and knockout (Clic4−/−) lines. Separate Clic4−/− were crossed to CD1 mice that overexpress Vegfa (Vegfhi) to create Clic4−/−;Vegfhi mice on a mixed C57BL/6J;CD1 background. Littermate Clic4−/−;Vegfhi and F2 C57BL/6J;CD1 served as CLIC4 knockout and WT controls, respectively. All experiments were approved by the University of North Carolina IACUC and performed in accordance with NIH guidelines.

Pial Collateral Morphometrics

P7-adult mice. After anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), the thoracic aorta was cannulated retrograde and the cortical pial circulation exposed via craniotomy. The vasculature was perfused with heparinized saline containing 40 μg/ml papaverine and 30 μg/ml sodium nitroprusside to fully dilate vessels, followed by a small bolus of Evans Blue (50 mg/ml in phosphate buffered saline) to stain vessel lumens. Yellow Microfil (FlowTech, Carver, MA) was then injected and allowed to cure. Brains were fixed with 4% paraformaldehyde (PFA) overnight and imaged with a stereomicroscope.

Middle Cerebral Artery Occlusion

A 4-mm incision was made just caudal to the right eye and the temporal muscle was retracted. A 2-mm2 cranial window was drilled (18000-17, FST, Foster City, CA) over the MCA trunk, which was cauterized (18010-00, FST, modified). The temporal muscle was resected and the incision closed. The brain was removed 24 h later and sliced into 1-mm coronal sections that were incubated in a 2% 2,3,5-triphenyltetrazolium chloride (TTC) in PBS at 37°C. Sections were coded to blind the investigator to genotype and imaged with a stereomicroscope (MZ16FA, Leica). Neocortical area and area devoid of TTC staining (infarct area) were measured (ImageJ) for each section, summed, and percent infarct volume of neocortex volume calculated.

Quantitative RT-PCR

Tissues were stored in RNA later (Sigma no. R0901) and pooled to produce one sample containing at least 6 embryos from at least 3 unrelated litters. Total RNA was isolated from the pia mater overlying the E18.5 dorsolateral collateral zone (see Ref. 21) or whole cortex. Reverse transcription was performed with SuperScript First-Strand Synthesis System (Invitrogen, no. 11904-018) following manufacturer instructions. Amplification was achieved with SYBR Green JumpStart Taq ReadyMix (Sigma, no. S4438) using StepOnePlus (AB Biosciences) and analyzed using the delta-delta CT method and StepOnePlus software. Gene expression was normalized to endogenous β-actin expression. Samples were analyzed in triplicate, and data from 3–4 RT-PCR samples were averaged.

Immunohistochemistry

Brains were fixed in 4% PFA and digested with 1 mg/ml collagenase (Sigma, no. C0130), and 100 μg/μl proteinase K (Qiagen, no. 19131) in PBS at 37°C for 20 min. Brains were permeabilized with 0.05% Triton (4°C overnight), blocked and exposed to anti-IgG IV (1:200, Millipore, no. AB756P) anti-VE-cadherin (1:300, BD, no. 55048) or anti-smooth muscle actin (SMA 1:300, Millipore no. 04-1094) at 4°C overnight. For confocal microscopy (Zeiss 510), vessels were secondarily stained using AlexaFluor-conjugated antibodies and the pia mater was gently separated from the cortex and mounted on slides. For smooth muscle actin quantification, the superficial cortex was dissected from the brain, gently weighted flat in a coverslip-bottom chamber and imaged (Lab-Tek no. 155380). For light microscopy, vessels were secondarily stained using biotin-conjugated horseradish peroxidase and DAB.

Aortic Ring Angiogenesis Assay

Six 0.5-mm thoracic aortic rings from each 4- to 6-wk-old mouse were serum-starved in Opti-MEM (Life Technologies, no. 31985-070) for 24 h and plated in 75 μl collagen type I (Millipore no. 08–115) (modified from Ref. 2). On day 6, rings were fixed and stained with anti-VE-cadherin (1:300, BD, no. 55048) and an AlexaFluor-conjugated secondary antibody. Sprout counts were obtained live on blinded samples using a dissecting microscope, and sprout length was determined using ImageJ.

Statistics

Values (means ± SE) were tested with independent t-tests or ANOVA followed by Bonferroni analysis (SPSS; PASW Statistics 18) with significance set at P < 0.05.
RESULTS

CLIC4 Deficiency Impairs Collateral Maturation but Not Collaterogenesis

To determine if CLIC4 deficiency in C57BL/6 mice affects collateral formation, we used immunohistochemistry to identify pial collaterals at E16.5 and E18.5. Embryonic arteries cannot be reliably filled with opaque solutions because they rupture under pressure. Thus lumen diameter, which is easily measured in the adult, cannot be reliably measured in embryos. Collateral number in Clic4+/− mice was similar to WT at both E16.5 and E18.5 (Fig. 1, A and B). We then examined collateral number and diameter in P7, P14, and adult mice after infusion of Microfil. Wild-type collateral number peaked at E18.5 and then declined slightly with pruning, consistent with previous reports (Fig. 1B) (5, 7). In contrast, Clic4+/− mice lost ~50% of their collaterals between E18.5 and P7, resulting in reduced number in the adult. Collateral diameter was smaller in Clic4+/− mice at all postnatal stages (Fig. 1C), consistent with our previous report for Clic4+/− mice on an outbred CD1-background (6). These results show that CLIC4 deletion has no effect on collateral formation but facilitates their pruning and, of the ones remaining, a failure to enlarge to normal diameter.

VEGF-A Overexpression Prevents Collateral Pruning in Clic4+/− Mice

VEGF-A induces EC sprout formation and promotes vascular maturation. In the cerebral vasculature, prolonged exposure to VEGF-A increases microvascular pericyte coverage and expression of the EC-percyte junctional protein N-cadherin (45). To test whether VEGF-A overexpression could restore collateral maturation in Clic4+/− mice, we crossed Clic4+/− with Vegfa-overexpressing mice to create Clic4+/−;Vegfa+/+ mice on a mixed C57BL/6-CD1 background. Isolated E18.5 pial meninges in CLIC4-deficient embryos indeed expressed less Vegfa than control mice, and levels were restored but not inflated by Vegfa overexpression. (Fig. 2A). Whole cortex homogenate showed no difference in Vegfa expression between the groups and may reflect tight compensatory Vegfa regulation in developing cortical neurons (Fig. 2B).

Vegfa-overexpression in Clic4+/− embryos did not alter collateral number at E18.5 (Fig. 2C). Consistent with Fig. 1, the mixed-background Clic4+/− mice lost ~50% of their collaterals between E18.5 and P7, and collateral number and diameter remained similarly low in adults (Fig. 2, B and C). In contrast to their Clic4+/− littermates, Clic4+/−;Vegfa+/+ mice did not lose collaterals; thus collateral number and diameter remained similar to WT in adults.

To confirm that existing collaterals pruned in Clic4+/− mice, we looked for evidence of “empty” vessel sheaths in the pia mater. Nascent vessels are known to secrete an extracellular matrix consisting predominantly of collagen IV that persists for weeks as an empty sheath after pruning. We immunostained isolated P4 pia mater for the EC marker VE-cadherin and collagen IV and all distal arteriolar-arteriolar collagen IV-positive/VE-cadherin-negative segments were counted. These sheaths were rare in WT, but present in every Clic4+/− pial membrane examined (Fig. 2E). When they did occur in Clic4+/− mice, the sheaths were almost exclusively located at the point of fusion with a larger opposing branch (Fig. 2E, arrows). The tapered appearance may be from stretching due to rapid growth of the neocortex at this time. The number of Clic4+/−;Vegfa+/+ sheaths was similar to WT (Fig. 2D) and is consistent with Fig. 2B showing no collateral pruning in this group.

CLIC4 Deletion Does Not Affect Endothelial Cell Sprouting

CLIC4 is required for normal tube formation, network branching and cell sprouting in isolated ECs in vitro and in tumor invasion and retinal growth assays in vivo (42, 43). Since collaterals are known to form by a sprouting-like mechanism from preexisting arterioles (21), we sought to determine if CLIC4 has a role in sprouting angiogenesis. Since there is no in vitro model of collateral formation, we used the aortic ring assay because it encompasses a complete “vascular niche” of interacting cell types and can be harvested from mice of different backgrounds and targeted genes. Background strain is known to affect angiogenic and collaterogenic potential (e.g., 4, 19, 32), and thus the aortic ring assay controls for any variation in pure C57BL/6 and mixed C57BL/6 × CD1 strains. Average sprout number and length were similar between both
Clic4 deletion. VEGF-A overexpression prevents pruning of nascent perinatal collaterals and deficiency in growth of diameter (D) in Clic4−/− mice. E: quantification of collagen IV-positive, endothelial cell-negative (VE-Cadherin-negative) “sleeves” (arrows) in P4 pial arterioles (representative confocal images; scale bar: 20 µm). Values are means ± SE; no. of animals given in columns. *P < 0.05; **P < 0.01; ns, not significant.

C57BL/6 WT and Clic4−/− mice (Fig. 3A), and the mixed-background WT and Clic4−/− mice (Fig. 3B). However, sprout number and length were larger in Clic4−/−;Vegfhi/+ rings, even though we did not see an increase in collaterals in Clic4−/−;Vegfhi/+ embryos. These results suggest that angiogenic sprouting potential may contribute to, but is not required for, collateralogenesis.

Overexpression of VEGF-A Prevents Worse Stroke After MCA Occlusion in Clic4−/− Mice

Differences in native collateral number and diameter closely correlate with (6, 7, 15, 33), indeed are causal for (33), differences in infarct volume. To determine if collateral number and diameter that were “rescued” by VEGF-A overexpression in Clic4−/−;Vegfhi/+ mice (Fig. 2) protects against ischemic damage, we performed MCA occlusion in mixed-background WT, Clic4−/− and Clic4−/−;Vegfhi/+ adult mice. Twenty-four hours after MCA occlusion, Clic4−/− had ~3-fold larger infarctions than WT mice (Fig. 4), confirming a previous report using CD1-background mice (6). In contrast, infarct volume in Clic4−/−;Vegfhi/+ mice was reduced to that in WT. Thus failure of proper collateral maturation in Clic4−/− mice (Figs. 1 and 2) causes larger infarctions, and both deficiencies are prevented by increasing VEGF-A expression.

Gene Expression Supports CLIC4 Requirement for Maturation of Nascent Collaterals

To determine if impaired pial collateral maturation is reflected in changes in expression of genes involved in stabilization, muralization, and growth of nascent vessels, the pial collateral zone [area of the pial membrane containing collaterals and distal branches of the ACA, MCA and PCA (Fig. 5A)] was harvested from P4 and P7 mice. We used C57BL/6 WT and Clic4−/− mice to avoid variability associated with crossing to an outbred strain (CD1). Expression of VEGFR1, VEGFR2 and VEGF-C were decreased in Clic4−/− mice while at P4, ADAM10 was significantly increased (Fig. 5B). Mural cell markers desmin, smooth muscle actin (SMA) and the smooth muscle-EC adhesion molecule N-cadherin were all lower at P7; angiopoietin-1 (Ang1), associated with vessel stabilization, trended lower. Other genes also associated with vessel maturation (Dil4, Hey1, Notch1, Tgfb1, Tgfbr2,
S1p1r, data not shown) did not show significant differences. Overexpression of VEGF-A in Clic4−/− mice restored altered gene expression to WT values, with the exception of Vegfr1 (Table 1). Expression of the other CLIC family member enriched in ECs, CLIC1, was similar in P4 WT and KO pial vessels (P = 0.78), consistent with baseline values previously observed in adult calf muscle (6).

Nascent Clic4−/− Collaterals Have Poor Mural Cell Coverage

Branches of the MCA and ACA trees (Fig. 6A, white arrows) are well-invested with SMA-positive cells by E14.5. Some nascent collaterals begin to attract SMA-positive cells shortly after formation (blue arrows) while some lack or have poor coverage (yellow arrows). By E18.5, distal-most arterioles of the MCA, ACA and the connecting collaterals are well-covered with SMA-positive cells (Fig. 6B, blue arrows), although occasionally a collateral can be found that is not (yellow arrow). In contrast, while SMA cell coverage is abundant in some Clic4−/− collaterals (Fig. 6C, blue arrows) in most it remains sparse (yellow arrows). Dual staining with the EC marker VE-Cadherin shows that some early postnatal Clic4−/− collaterals never acquire substantial SMA coverage (Fig. 6D, white arrows), which may facilitate pruning. Quantitation of collateral diameter from the VE-Cadherin signal and mural cell coverage from the SMA signal reveals that the average

**Fig. 4.** VEGF-A overexpression in Clic4−/− mice prevents increased infarct volume. Staining with 2,3,5-triphenyltetrazolium chloride 24 h after permanent middle cerebral artery occlusion. Values are means ± SE; no. of animals given in columns. **P < 0.01.**
collateral is already smaller in diameter with less SMA coverage in P4 Clic4−/− pups (Fig. 6E and F). VEGF-A overexpression increases both collateral diameter (Fig. 6E) and SMA-positive cell coverage (Fig. 6F), presumably above some threshold that favors pruning (Fig. 2C). A scatterplot illustrates the collateral maturation deficiencies in Clic4−/− pups (Fig. 6G).

**DISCUSSION**

Preexisting collaterals are important determinants of tissue survival after arterial occlusion. However, our understanding of how they form, mature and persist throughout a lifetime is limited. The present study demonstrates that CLIC4 is required for early postnatal maturation of newly formed collaterals. Within the first postnatal week, mice lacking CLIC4 lose 50% of the collaterals that form during embryogenesis, and those that remain grow to a smaller diameter. Both deficiencies persist unchanged in the adult, resulting in a threefold larger infarct volume after MCA occlusion. The role of CLIC4 in collateral maturation involves an interaction with VEGF-A signaling. During the maturation phase in Clic4−/− mice, expression of VEGF receptors and mural cell marker genes is reduced. Furthermore, VEGF-A overexpression normalizes most of these expression deficits, prevents both the defects in collateral maturation and increased stroke severity in the adult.

We previously reported that fewer pial collateral were present on P1 in Clic4−/− mice and suggested that CLIC4 deficiency impairs collaterogenesis (6). However, this conclusion remained uncertain because in wild-type mice collateral maturation begins after the maximal number of collaterals have formed by E18.5, followed by slight pruning that is evident by even P1 (6, 10). More detailed study of CLIC4 in the present study, including in two different strains of mice, indicates that CLIC4 deficiency does not affect initial collaterogenesis. Moreover and unlike in the previous study, embryonic stage-matched littersmates were examined. This is important because the maturation state of mice at birth can vary. Typical gestation is 21 days yet parturition can occur between E19.5 and 22.5, depending on litter size, cage density, strain, and other factors. With perinatal collateral pruning, a differing number of gesta-

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**Table 1.** Vegfa overexpression restores gene expression, except Vegfr1, to WT values in the P4 pial vasculature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Vegfr2</td>
<td>1.00 ± 0.33</td>
<td>1.18 ± 0.24</td>
</tr>
<tr>
<td>Vegfr1</td>
<td>1.00 ± 0.09</td>
<td>0.69 ± 0.34*</td>
</tr>
<tr>
<td>Vegfc</td>
<td>1.00 ± 0.07</td>
<td>1.21 ± 0.29</td>
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<tr>
<td>Ang1</td>
<td>1.00 ± 0.29</td>
<td>1.71 ± 0.18</td>
</tr>
<tr>
<td>Adam10</td>
<td>1.00 ± 0.13</td>
<td>1.25 ± 0.29</td>
</tr>
<tr>
<td>SMA</td>
<td>1.00 ± 0.13</td>
<td>1.64 ± 0.43</td>
</tr>
<tr>
<td>Desmin</td>
<td>1.00 ± 0.57</td>
<td>2.21 ± 1.33</td>
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Values are group means ± SE; n = 3 for each group. Each sample comprised pooled pial membranes of 3–6 individual mice; P values from 2-tailed independent t-test. P4, postnatal day 4; WT, wild type. *P < 0.05.
tional days between E18.5 and birth could account for differences in collateral number measured on P1.

Endogenous CLIC4 expression patterns in the developing and adult mouse have only recently been described after the development of a knockin Clic4 green fluorescent protein mouse model (29). A midgestation, CLIC4 is expressed widely throughout the embryo and is particularly strong in the brain. At E13.5, after the pial vascular plexus has formed but before collaterogenesis begins, CLIC4 expression is strong in the entire forebrain. By E15.5, expression drops in areas of the
brain but remains strong in the neocortex. It then declines through E18.5. While the authors did not specifically examine or discuss CLIC4 expression in pial or other cerebral vessels in embryos or adults, they state that CLIC4-GFP expression was uniformly present in vascular smooth muscle cells and endothelium in virtually all adult tissues. This observation suggests that CLIC4 is constitutively present in blood vessels but is actively reduced in parenchymal cells. Confirmation of this assumption is currently hindered by absence of specific antibodies (29). Such a decline in CLIC4 expression in parenchymal cells in the vicinity of newly formed collaterals may serve as a paracrine signal that initiates mural cell recruitment and/or differentiation. Therefore, when CLIC4 is absent, this disrupts collateral maturation which is what we observed. This hypothesis is consistent with CLIC4’s interaction with the TGFβ signaling pathway (see below).

In contrast to collateral maturation, we found that Clic4 deletion had no apparent effect on formation of the embryonic pial plexus or its remodeling into artery and vein trees. Deletion also had no effect on angiogenesis in the aorta ring assay. Consistent with these findings, a previous study (43) found that formation of the outer retinal plexus appeared normal in Clic4−/− mice during (P4) and near the end (P7) of the radial outgrowth phase, a phase dominated by angiogenic sprouting and capillary plexus formation (9, 25), although the leading edge of the plexus tended to reach the outer retinal margin slightly later. However, on P21 the mature capillary bed had a lower number of branch points and overall vessel density (43). Maturation of the early retinal plexus into artery and vein trees and intervening capillaries involves selective pruning and re-patterning processes that occur concurrently with radial outgrowth and are complete by P15 (9, 25). Our observation that CLIC4 is required for pruning and maturation of the nascent collateral circulation has analogy to the deficiencies in retinal vascular remodeling (43).

At variance with these findings are studies using cultured ECs (3, 42, 43). CLIC4 knockdown inhibits while overexpression promotes proliferation. Level of CLIC4 expression also affects organization into plexus-like structures and formation of intracellular vacuoles and lumenization, but not migration of HUVECs (42). A number of factors could contribute to the differences seen in vivo, in explant, and in vitro, most prominently that HUVEC cultures lack the presence of other cell types present in the vascular wall. In contrast, the ring assay provides ECs, at least initially, in their normal proximity to adjacent mural cells, which may better approximate the in vivo setting for ECs and any reliance on CLIC4 for angiogenic activities. Also, “neovessel” growth begins approximately 2–3 days after the rings have been placed in culture and continues to 6–7 days (1). Thus early outgrowth from aorta rings of Clic4−/− mice might not be expected to be altered since CLIC4 does not affect endothelial cell migration, and sprout length and number are in part based on EC migration. Moreover, since increasing environmental VEGF-A is well known to intensify the angiogenic response in cell culture and aortic ring assays (47, 48), that it did so in the Clic4−/−;Vegfpho+ rings served as a control for the assay.

Our observation that VEGF-A overexpression can rescue the deficits incurred by Clic4 deletion suggests that CLIC4 and VEGF-A interact to drive maturation of nascent collaterals. Although little is known about how CLIC4 and VEGF-A interact, evidence suggests TGFβ signaling may be involved. CLIC4 is both regulated by and is an enhancer for TGFβ signaling (36, 37). After TGFβ receptor/ALK5 activation, CLIC4 complexes with transcription factor Smad2/3 and translocates to the nucleus, where the complex protects against deactivation of TGFβ-induced phosphorylated Smad2/3, thereby enhancing TGFβ signaling (37). TGFβ family members are known to regulate VEGF-A expression via Smad proteins (especially Smad2, 3 and 4, depending on cell type) and Wnt/Beta-catenin signaling (8, 16, 18, 27). In turn, VEGF-A regulates CLIC4 expression in both a positive and negative manner: VEGF-A-activated ECs downregulate Clic4 during tubular morphogenesis in vitro but strongly upregulate Clic4 in developing embryoid body-derived and pathological angiogenesis (3). Given the above interactions, in the present study, deletion of Clic4 may have reduced, but not fully blocked, TGFβ activity and subsequent VEGF-A signaling. This could account for the reduced Vegfr1 and Vegfr2 expression that we observed in the collateral zone of the pia mater of Clic4−/− mice where pruning was accentuated, and the ability of VEGF-A overexpression to rescue collateral loss. Vegfr1 largely, but not exclusively, functions as a VEGF-A “sink.” That Vegfr2 expression was restored in Clic4−/−;Vegfpho+ mice, but not Vegfr1 expression, suggests upregulation of Vegfr2 with Vegfa overexpression. This feed-forward effect of VEGF-A in vivo has been reported previously (12).

Deficiency in outward remodeling of collaterals after MCAO is unlikely to underlie the greater infarct volume in Clic4−/− mice. It is well established that preexisting collateral number and diameter in animal studies and collateral score (“status”) strongly correlate with core infarct volume measured 24 h after proximal occlusion of the MCA (6, 7, 20, 35, 46). Whether outward remodeling of preexisting collaterals can occur fast enough to rescue penumbra has not been determined. In a study of the time course for remodeling of pial collaterals in the mouse after permanent MCA occlusion, significant anatomic lumen enlargement of pial collaterals, a process that requires EC and SMC proliferation and restructuring of ECM, was first evident at 36 h (46). Much previous evidence in animals and humans suggests that final core volume is established well before this time (11, 41), although some penumbra might be evident at later stages in certain individuals.

We found that Clic4−/− collaterals are invested with fewer SMA-positive mural cells at E18.5, and that the pial membrane containing collateral vessels has lower expression of marker genes for mural cells. Mural cell coverage remains low through P4, when collateral diameter is measurably smaller in CLIC4-deficient mice. These data are consistent with a report demonstrating that CLIC4 deficiency halts vascular smooth muscle cell migration by interfering with intracellular RhoA and Rac1 signaling (38). Collaterals form later than the general cerebral vasculature and because they are not required for survival, they may be prone to pruning if mural cell coverage is delayed. Our results also support our previous data showing that CLIC4-deficiency results in pruning of collaterals but not neighboring distal-most arterioles (6). A striking difference between distal-most arterioles and collaterals is that the former experience unidirectional blood flow while the latter experience little net blood flow and thus very low shear stress (5). Shear stress induces TGFβ signaling in ECs by activating its receptor ALK5 (44) and downstream Smad2 (13, 14). During develop-
ment, Smad2/3 activation is not required for embryonic angiogenesis but it is indispensable for the recruitment of mural cells to nascent vessels and subsequent vascular maturation (17). Since CLIC4 enhances Smad2/3 signaling (37), CLIC4-deficient collaterals, vessels that are under low shear stress and thus perhaps low TGFβ receptor activation, may be unable to recruit mural cells at the same rate as neighboring distal-most arterioles and thus more prone to destabilization and pruning. The mechanism by which CLIC4 mediates smooth muscle cell investment in collaterals remains to be discovered.

We also observed that Adam10 expression was increased in P4 CLIC4-KO pial membranes. ADAM10 is a membrane-anchored sheddase that cleaves proteins from the cell membrane, and is well known for its role in cleaving Notch to form the bioactive notch intracellular domain. We previously showed that endothelial-specific knockout of ADAM10 during collaterogenesis inhibits collateral formation (21). In perivascular cells, ADAM10 is known to cleave platelet-derived growth factor receptor β (PDGFRβ), a tyrosine kinase receptor activated by PDGF (23) that promotes pericyte recruitment to nascent vessels and differentiation. However, the authors were unable to show a regulatory role for ADAM10 in PDGFRβ signaling in vitro. It is possible that Adam10 expression is increased in vivo during collateral pruning as a compensatory mechanism to enhance Notch signaling, active endothelial cell sprouting and PDGF secretion, or is enhancing PDGFRβ shedding and therefore defective mural cell investment. Studies determining which cell types are responsible for the increased pial Adam10 expression would help interpret this result.

There are several factors that hinder studying the interaction between CLIC4, VEGF, and TGFβ in collaterogenesis. The major one is that there is no in vitro model of collaterogenesis, in part because of the inherent complexity of the cell biology of the process, but also because collaterogenesis, itself, was only recently described (21). Nascent collaterals form late during embryogenesis and experience cyclic mechanical forces from blood pressure and fluid shear stress that, thus far, cannot be recapitulated in vitro. Therefore, new methods will be required to permit selective in vivo transfection of expression vectors into ECs and other cells involved in collaterogenesis at the proper developmental stage. Another factor is that collaterals must be identified in situ; collaterals by definition cross-connect arterial trees. Therefore, immunohistochemical examination of protein localization and expression must be performed on whole mount tissue, which can hinder antibody penetration. Last, we used mice with a global deletion of CLIC4 because no floxed line was available. However, a mouse line with a floxed CLIC4 allele has been recently described (30), which will aid future studies.

In conclusion, collateral number and diameter in the adult depend not only on embryonic collateral formation but also perinatal collateral maturation. CLIC4 is not required for collaterogenesis but is necessary to prevent pruning and promote growth of collateral diameter to their normal dimensions during maturation. CLIC4-deficient mice thus have fewer, smaller-diameter collaterals in the adult and experience more severe strokes. CLIC4’s role in collateral maturation involves interactions with VEGF-A, as overexpression of VEGF-A in Clic4<sup>−/−</sup> mice prevents the deficiencies in both collateral extent and stroke severity. A better understanding of the process of collateral maturation, as well as whether altered physiological conditions or pathological processes can adversely affect it, are important directions for future work.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.L.L. conception and design of research; J.L.L. and N.J.T. performed experiments; J.L.L. and N.J.T. analyzed data; J.L.L. and N.J.T. interpreted results of experiments; J.L.L. prepared figures; J.L.L. drafted manuscript; J.L.L. and J.E.F. edited and revised manuscript; J.L.L. and J.E.F. approved final version of manuscript.

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