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ORIGINAL ARTICLE

Genetic and Environmental Contributions to Variation in the Posterior Communicating Collaterals of the Circle of Willis

James E. Faber[1] · Hua Zhang[1] · Wojciech Rzechorzek[1] · Kathy Z. Dai[1] · Benjamin T. Summers[1] · Cooper Blazek[1] · Samuel J. Hedges[1]

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Abstract

Variation in blood flow mediated by the posterior communicating collateral arteries (PComs) contributes to variation in the severity of tissue injury in obstructive disease. Evidence in animals and humans indicates that differences in the extent of PComs, i.e., their anatomic lumen diameter and whether they are present bilaterally, unilaterally, or absent, are a major factor. These differences arise during development since they are present at birth. However, the causal mechanisms are unknown. We used angiography after maximal dilation to examine involvement of genetic, environmental, and stochastic factors. The extent of PComs varied widely among seven genetically diverse strains of mice. Like pial collaterals in the microcirculation, aging and hypertension reduced PCom diameter, while in contrast, obesity, hyperlipidemia, metabolic syndrome, and diabetes mellitus had no effect. Naturally occurring intrauterine growth restriction had no effect on extent of PCom or pial collaterals in the adult. The number and diameter of PComs evidenced much larger apparent stochastic-dependent variation than pial collaterals. In addition, both PComs underwent flow-mediated outward remodeling after unilateral permanent MCA occlusion that varied with genetic background and was greater on the ipsilesional side. These findings indicate that variation in the number and diameter of PCom collateral arteries arises from stochastic factors and naturally occurring genetic variants that differ from those that cause variation in pial collateral arterioles. Environmental factors also contribute: aging and hypertension reduce PCom diameter. Our results suggest possible sources of variation of PComs in humans and provide information relevant when studying mouse models of occlusive cerebrovascular disease.

Keywords Collateral circulation · Posterior communicating artery · Circle of Willis · Genetics · Hypertension · Aging

Abbreviations

ACA	Anterior cerebral artery
BA	Basilar artery
CoW	Circle of Willis
CSRFs	Cardiovascular and stroke risk factors
E1	Embryonic day 1(2,3…etc)
ICA	Internal carotid artery
MCA	Middle cerebral artery
pMCAO	Permanent proximal M2-MCA occlusion P1 postnatal day 1(2,3…etc)
PCA	Posterior cerebral artery
PCom	Posterior communicating collateral artery
RTG	Renin overexpressing transgenic mouse model of hypertension
SGA	Small for gestational age
WT	Wildtype strain

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✉ James E. Faber
jefaber@med.unc.edu

[1] Department of Cell Biology and Physiology, McAllister Heart Institute, University of North Carolina, 6309 MBRB, Chapel Hill, NC 27599-7545, USA

Introduction

The circle of Willis (CoW) is a specialized network created by three collateral arteries, the anterior communicating artery (ACom) and the bilateral posterior communicating arteries (PComs) that interconnect the internal carotid arteries (ICAs) and basilar artery (BA) via the proximal trunks of the anterior and posterior cerebral arteries. Physiologically, the CoW has

been suggested to dampen normally occurring transient differences in pressures and flows in the ICAs and BAs [1], while in pathological conditions, it provides alternative pathways for perfusion when atherosclerotic, thrombo-embolic, hemorrhagic, and congenital obstructions occur within or proximal to the CoW [2–5]. Unfortunately, the presence and diameter of the ACom and especially the PComs display remarkably wide variation, as seen on angiographic imaging of patients with cerebrovascular disorders and in cadaveric studies of individuals free from cerebral disease [2–20]. Incidence of hypoplastic (small lumen diameter) or absent PComs averaged 47% in the above-referenced cadaveric studies, with similar findings in the angiographic studies. For example, in the largest CTA study to date [7], incidence of bilateral or unilateral absence of the PComs averaged 17 and 16%, respectively, and ACom absence averaged 1%. The variation in PComs is attributed to developmental differences since it is present at preterm and birth [3, 21–29]. However, the cause of the variation, i.e., whether it is due to differences in genetic, environmental, or random/stochastic factors, arising during development has not been investigated. Understanding this is important not only from a basic science standpoint but also because deficient PComs impact surgical decision-making and increase the risk of TIA, ischemic stroke, stroke reoccurrence, severity and poor outcome, and incidence of subarachnoid hemorrhage [2–5, 12, 14, 30–34]. Regarding the latter, the most frequent sites of intracranial aneurisms, accounting for 43% in a recent study [35], occur at end-segments of the ACom and PComs. Thus, a variant CoW has recently been proposed as a risk factor for the occurrence and severity of both ischemic and hemorrhage stroke [2, 34–38].

The diameter and number of PComs (i.e., whether they are present bilaterally, unilaterally, or absent) also appear to vary widely in different mouse strains, suggesting involvement of genetic factors [39–48]. However, there is considerable disagreement among studies even for the same strain. For example, PComs in C57BL/6 mice were reported to be bilateral and well developed [42, 43], or bilateral and hypoplastic [44, 46], or highly variable in number [48]. Unfortunately, previous studies did not use perfusion fixation at maximal dilation and often relied on small sample sizes or did not quantify PCom number and/or diameter. Rats have been reported by some but not all investigators to have bilateral well-developed PComs, although only two strains have been studied [49]. We have shown that the number and diameter of pial (leptomenigeal) collateral arterioles vary widely among 22 mouse strains, resulting in large differences in infarct volume after permanent middle cerebral artery occlusion (pMCAO) [50, 51]. Polymorphic forms of a novel gene, *Rabep2* [52, 53], and associated signaling pathway elements [54] have been shown to be responsible for most of this variation. However, whether variation in PComs follows the same strain-specific pattern and dependence on variant forms of *Rabep2* has not been examined.

Environmental factors could also contribute to PCom variation. In mice, aging, hypertension, metabolic syndrome, and other cardiovascular-stroke risk factors (CSRFs) cause pruning away of pial collaterals and a smaller diameter in those that remain [48, 55–58]. Findings for pial collateral score in humans are consistent with this for aging and metabolic syndrome [59, 60]. As well, the incidence of deficient PComs in patients has been associated with hypertension [12, 61, 62]. In addition, unilateral and bilateral absence of PComs increases with aging in cadaveric studies of humans lacking cerebral disease [7] and in angiographic studies of patients with cerebrovascular disease [11, 63]. However, whether these findings reflect the concomitant presence of other CSRFs is not known. It is also possible that adverse maternal or in utero conditions, for example, those that cause intrauterine growth restriction (i.e., small for gestational age, SGA) [64–67], could impact the development of PComs. Interestingly, the incidence of ischemic stroke and poor outcome has been associated with SGA, independent of the presence of hypertension [66, 67]. Stochastic or random variation during development that is not dependent on genetic or environmental factors could also contribute to variation in PCom collaterals. For example, large palmar arch collaterals present in the one hand can be absent in the other hand of the same individual [68]. No experimental or human studies have examined whether aging, other CSRFs, SGA, or stochastic factors contribute to variation in PComs.

The purpose of this study was to examine the contribution of genetic factors (i.e., differences in genetic background, deletion of *Rabep2*), environmental factors (i.e., age, presence of CSRFs, SGA), and stochastic factors to variation in the extent (i.e., number and diameter) of the PCom collateral arteries and, where relevant for comparison, to variation in the extent of the pial collateral arterioles. We did not examine the AComs because of the difficulty in imaging the anterior intercerebral fissure in mice and because AComs evidence much less variation in humans and mice (i.e., the above references report them as absent in 0–3%). A second aim was to determine if flow is induced across the PComs after occlusion of the MCA—the most common cause of large-vessel ischemic stroke. It is generally assumed that PCom flow, which is normally low or absent due to lack of a pressure difference [1], is not or minimally recruited when the site of occlusion is distal to the CoW [1, 67–69]. However, no studies have examined this question.

Methods

See the Online Supplemental Resource for details. Mouse strains compared for the effect of genetic background on PComs were 3–5 months of age. Genetic mouse models of CSRF presence were on the C57BL/6J (B6) background; mice were fed normal chow, were 8 ± 0.75 months of age unless

indicated otherwise, below, and had the following mutations: obesity (*Lep*$^{ob/ob}$), hyperlipidemia (*Apoe*$^{-/-}$), metabolic syndrome/type 2 diabetes (*Lep*$^{ob/ob}$;*Ldlr*$^{-/-}$, examined at 6 months of age), and type 1 diabetes (*Ins2*^{Akita}). These mutants were compared to B6 wildtype mice (WT). Mice with hypertension (renin transgenic, RTG$^{+/-}$ and RTG$^{+/+}$, on a mixed B6;129SvEvTac background) were compared with littermate WT controls. Baseline characteristics of the above mice are in the Supplement. All studies used male mice unless indicated otherwise in the figure legends. The PCom diameter and number did not differ with sex in B6 mice, although there was a trend for larger diameter and number in females [69]. The mouse PCom, as defined herein, is in the anatomic location of the P1 segment of the PCA in humans (visa versa for the human PCom) (see Fig. 1)—definitions based on diameter and function that are also used in most previous mouse studies, e.g., [41, 42]. Angiography was performed after maximal dilation, fixation, and filling with latex. Viscosity and pressure were adjusted to fill the arterial and collateral circulations while minimizing capillary transit and venous filling. All pial collaterals between the ACA and MCA trees of both hemispheres were identified, and their lumen diameters at midpoint were determined and averaged for each animal. Diameter of PComs and primary intracranial arteries were measured at defined distances from bifurcating junctions (see Supplement). PCom remodeling (anatomic lumen enlargement) was measured 6 days after unilateral pMCAO of the proximal M2-MCA. For analysis of the effect of intrauterine growth restriction, neonates from seven different B6 breeder pairs were weighed on postnatal day (P) P1–P2, P24–26, and

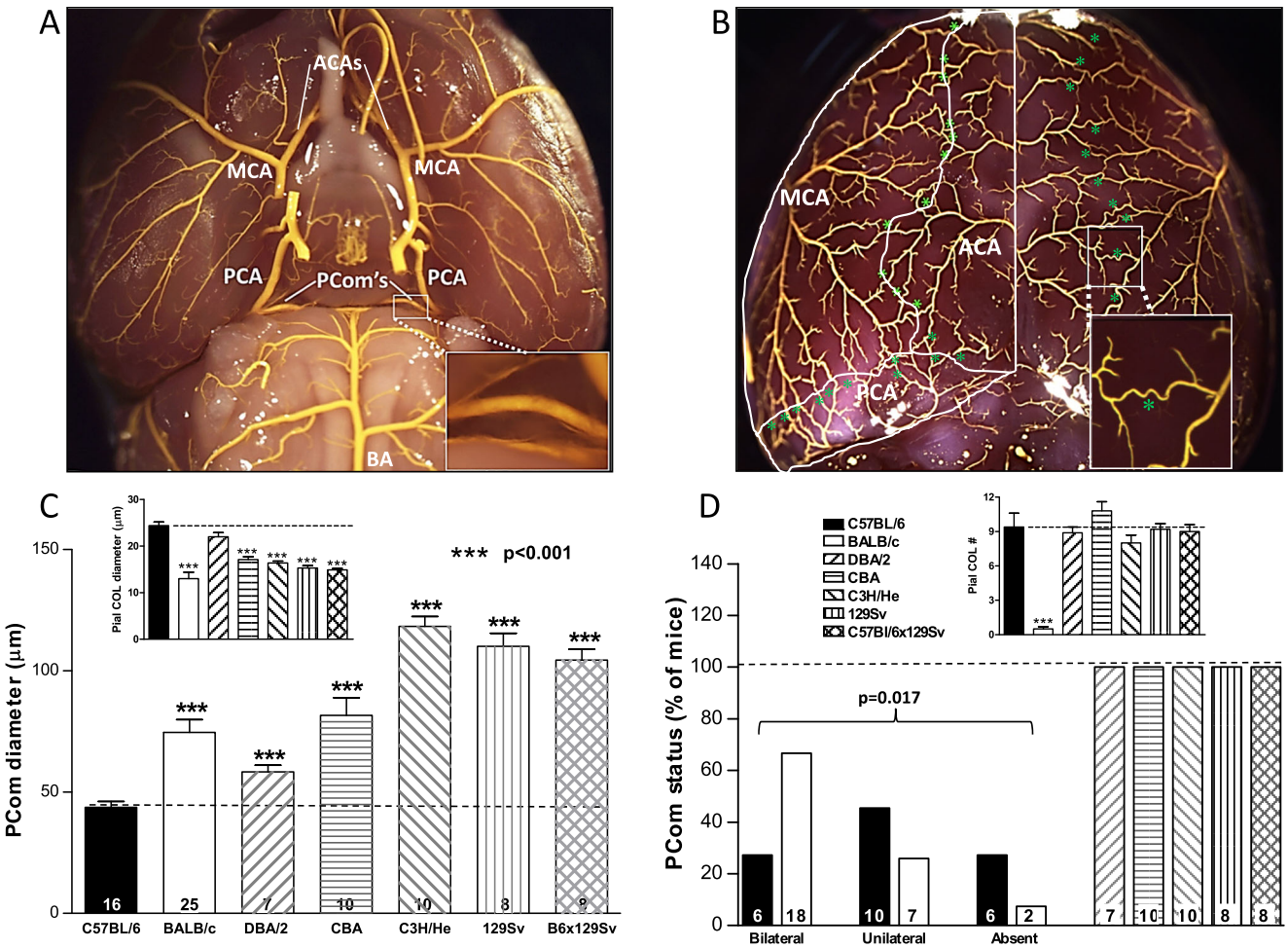

Fig. 1 Diameter and number of PCom collaterals vary widely with genetic background. **a**, **b**, Pial arterial vasculature filled with Microfil^{R} after maximal dilation and fixation; brain was counterstained with Evans blue. The rodent posterior circle of Willis (CoW) has the human "fetal" pattern; thus, PComs correspond to P1-PCAs in humans. Green stars, pial collaterals (COL). Dorsal aspects of the MCA, ACA, and PCA tree territories are outlined in the left hemisphere. The strain-specific pattern of differences in PCom diameter (**c**) and number (**d**, i.e., present bilaterally, unilaterally, or absent) does not follow the pattern of differences for pial collaterals (inset graphs modified from data presented in Zhang et al. [50]), e.g., PComs of C57BL/6 are larger in diameter and more frequently absent or unilaterally present than in BALB/c (two-tailed X^2 test). Thus, different polymorphisms underlie variation in PCom and pial collaterals. See Table I (Supplement) for PCA diameter, brain weight, and other parameters. The values are mean ± SEM, and numbers in base of bars are number of animals in this and subsequent figures and tables, unless indicated otherwise. ***p 0.001, two-tailed t tests vs. C57BL/6. 3–5-month-old male mice

at 10 weeks of age when angiography was performed. Pups with body weights that were ≤ 12.5% of the average of all of the pups on P1–P2 were defined as having intrauterine growth restriction/small for gestational age (SGA). The STAIR criteria adhered to in this study, including blinding to group before conducting morphometry and no exclusion of data points, are in Supplement. Significance ($p < 0.05$) was tested using *t* tests, ANOVA followed by Bonferroni *t* tests, linear regression, chi-square, Fisher's Exact, and Modified Leven's (Brown-Forsyth) tests.

Results

PCom Number and Diameter Vary Widely with Differences in Genetic Background

The extent (number and diameter) of PComs in B6 mice was the smallest among seven strains (Fig. 1). The number varied greatly within the B6 and BALB/c strains, suggesting that stochastic/random factors can affect PCom formation (addressed below). The diameter was greater in four out of five of the other strains, and all five had bilateral PComs. PCom length was short in CBA, intermediate in C3H/He, and long and arching in the other five strains, but was not quantified because it has a small effect on conductance compared to diameter and number. In an F1 cross that we constructed, the 129Sv background exerted dominance over the B6 background for PCom number and diameter, further confirming the strong influence of genetic background on PCom phenotype. Interestingly, dominance of the 129Sv genome was also evident for PCA diameter, PCA territory, and pial collateral diameter (Supplemental Table I).

In agreement with a previous study [50], territory of the ACA, MCA, and PCA trees varied with genetic background (Table I); however, the variation was much smaller than the variation in the extent of PCom or pial collaterals. Because the PComs interconnect the BA and PCAs in rodents, strains with larger PCA trees may secondarily have larger PCom diameters. However, while PCom diameter correlated with PCA territory among the seven strains, the relationship was negative (Table I). This could reflect that strains with larger MCA and ACA territories combined, and thus, smaller PCA territories [50]—which may arise from genetic background-dependent differences in the time-course of growth of regions of the neocortex and their arterial trees during development [3, 20, 21, 24–26]—may promote flow-mediated outward remodeling of the PComs to enable the pressure and flow in the BA to provide a larger contribution to the ICAs for perfusion of the MCA and ACA "anterior" territories. Altered branching structure in the trees and/or greater autoregulatory microvascular tone in strains with smaller PCA territories may offset their larger PCA diameters to maintain normal capillary pressure and flow. Consistent with this hypothesis, PComs were bilaterally present in these strains. PCom diameter did not correlate with pial collateral number, brain weight, or body weight (Table I).

It is also possible that strain-dependent variation in PComs reflects the effect of functional polymorphisms in genes in the signaling pathway (which is unknown) that governs their formation. Previous studies found that the extent of pial collaterals varies by 56-fold among 22 strains that include the above six inbred strains (with BALB/c having the lowest and B6 nearly the highest extent) [50, 51]. Most of this variation is caused by coding variants in *Rabep2*—a critical gene in the pathway that drives formation of pial collaterals late in gestation and determines their

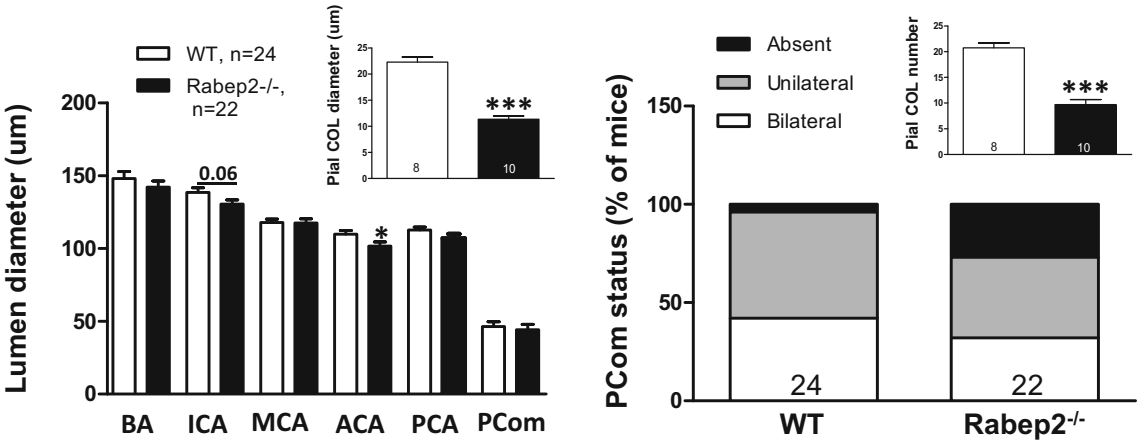

Fig. 2 Deficiency of Rabep2 does not alter the extent of PCom collateral arteries. By comparison, inset graphs show decrease in pial collateral extent in *Rabep2* deficient mice (data from Lucitti et al. [53]). WT (C57BL/6) and Rabep2$^{-/-}$ do not differ, respectively, for brain weight (g, 578 ± 8, 599 ± 8), body weight (g, 26 ± 0.7, 27 ± 0.9), or brain/body weight (0.0228 ± 0.0009, 0.0226 ± 0.0008). PCom status is not different by two-tailed X^2 test ($p = 0.49$). Two-tailed *t* tests for diameter. 3–4-month-old male mice

extent in the adult [53]. Among the strains in Fig. 1c, d, the pattern of variation in the extent of PComs is opposite of that for pial collaterals (insets in Fig. 1c, d). The PCom extent was unaltered in B6.*Rabep2*$^{-/-}$ knockout mice that have significantly reduced pial collateral extent (Fig. 2). The PCom diameter correlated positively with PCA diameter, but not diameter of the other primary intracranial arteries (Supplemental Fig. I). The above findings showing the absence of association of PCom and pial collateral phenotypes among the strains and lack of effect of loss of *Rabep2* on PCom extent indicate that genetic-dependent variation in PCom collateral arteries and pial collateral arterioles are governed by different polymorphic genes and suggest that different signaling pathways mediate their formation.

Infarct volume after pMCAO does not correlate with differences in PCom number and diameter in the strains studied in Figs. 1 and 2, but instead correlates closely with their differences in pial collateral number and diameter since the occlusion is distal to the CoW [50, 53].

Aging and Hypertension, but Not Other Cardiovascular/Stroke Risk Factors, Affect PCom Diameter

Genetic and environmental factors vary among stroke patients, and the presence of CSRFs is known to associate with stroke severity and outcome. In mice, aging (≥ 16 months of age), and 6–8 months of exposure to hypertension, metabolic syndrome, hyperlipidemia, obesity, and diabetes mellitus (using the genetic mouse models shown in Fig. 3a), causes rarefaction of pial collaterals (loss of number and/or decline in diameter) and increased infarct volume after pMCAO [55–58]. We thus examined the effect of these models of CSRFs on PCom collaterals to determine if similar effects occurred. The diameter declined progressively with modest

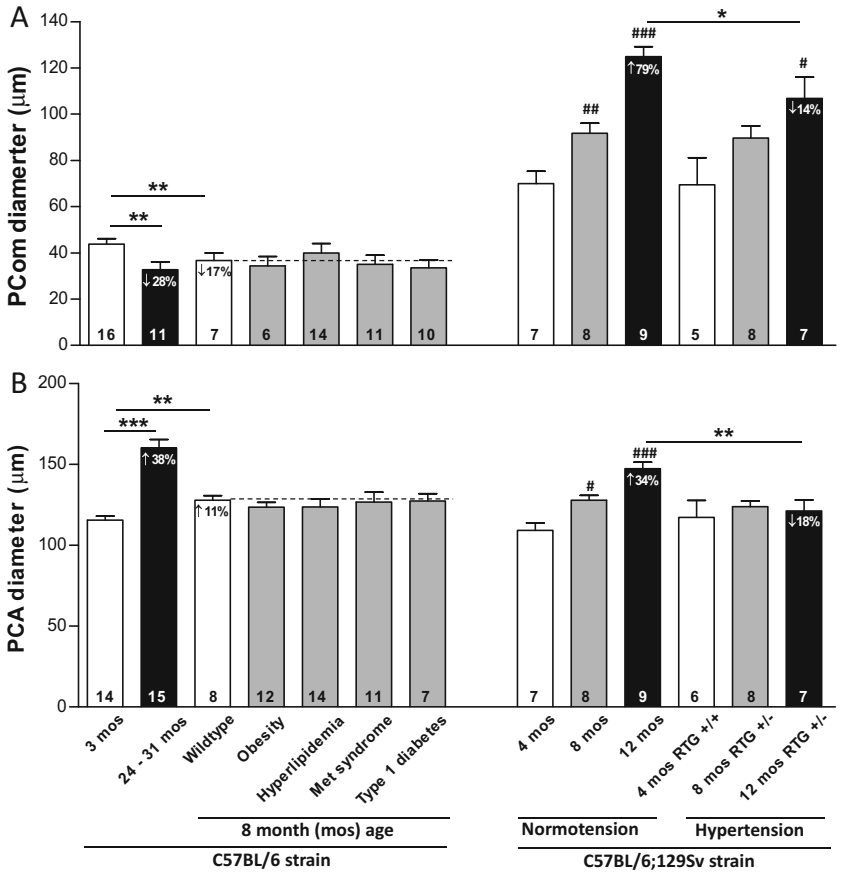

Fig. 3 Effect of cardiovascular/stroke risk factors (CSRFs) on PCom and PCA diameter. **a** With aging, PCom diameter declined in C57BL/6 strain but increased in C57BL/6;129Sv strain. Sustained hypertension through 12 months of age reduced PCom diameter. Mean arterial pressure is elevated ~43, 30, and 30 mmHg in renin-dependent (RTG) hypertensive mice at the three ages [57]. Other CSRFs had no effect (obesity, 2-fold increased body weight; hyperlipidemia, 4-fold increased cholesterol; metabolic syndrome, increased body weight, glucose, and cholesterol [57]). **b** PCA diameter increased with aging in both strains and was reduced by hypertension of 12 months duration. Other CSRFs had no effect. PCom number (bilateral, unilateral, absent) was not different (Supplemental Figures I, II). By comparison, pial collateral number and diameter evidenced age dose-dependent declines beginning at 16 months of age in normotensive C57BL/6 mice, in the above RTG hypertensive mice, and in the other four CSRF mice [57]. Data for PCom number are in Supplemental Figures I, II. *, **, ***$p < 0.05$, 0.01, 0.001; #, ##, ###$p < 0.05$, 0.01, 0.001 versus 4-month-old normotensive or hypertensive mice. No significant differences were observed between sexes

(8 months) and advanced aging (24–31 months) in B6 mice (Fig. 3a). However, the opposite was seen in mixed B6;129Sv mice. In the latter, hypertension of 12 but not 4 or 8 months duration caused a decrease in PCom diameter. Arterial pressures at 4, 8, and 12 months were 43, 30, and 30 mmHg above wildtype (Supplement). Modest and advanced aging were accompanied by increased PCA diameter (Fig. 3b), suggesting that the changes in PCom diameter were not simply secondary to changes in (i.e., "following") PCA diameter or visa versa. Like PCom, PCA diameter was decreased in 12-month-old hypertensive mice. Other CSRFs had no effect on PCom or PCA diameters. The PCom number was not significantly affected by aging or any CSRFs (Supplemental Figure II), although small sample sizes make this conclusion uncertain.

Intrauterine Growth Restriction Has no Effect on PCom or Pial Collaterals

PCom number varied markedly among individual mice within the B6 and BALB/c strains despite having the same genotype, age, and vivarium environment (Fig. 1d). Furthermore, comparison of Figs. 1, 2, 4 and Supplemental Figures II and III shows that the pattern of variation in PCom number varies greatly among five random samples of B6 mice (all are 2.5–5 months of age except Figure III which are 8 months of age). In addition, PCom diameter varied widely and randomly between the two hemispheres in the seven strains in Fig. 1 (data not shown; note, average diameter for right versus left sides did not differ for a given strain). Although these variations appear stochastic, it is possible that inter-individual differences in environmental factors present in utero might contribute to the high variability. In support, incidence of ischemic stroke and poor outcome associate with low birth weight in humans [65–67], which is also termed intrauterine growth restriction or small for gestational age (SGA) when not due to preterm delivery. Therefore, we examined the effect of naturally occurring SGA [70, 71] in B6 mice, defined as newborn pups within a litter weighing in the lower 12.5 percentile (i.e., "runts"). The PCom diameter and number of 10-week-old adults that were SGA at birth were similar to mice with normal birth weight (Fig. 4). There were no sex differences in PComs, although small *n* sizes weaken the comparisons. The extent of pial collaterals was also unaffected by SGA (Fig. 4), as expected since pial collaterals did not display unusually large variability. Supplemental Table II: SGA mice evidenced catch-up growth of brain weight by adulthood. Both brain weight and PCA diameter of adult SGA mice trended toward being smaller, although PCA diameter did not correlate with birth weight. Like that in 10% of humans with SGA who do not show catchup growth in body weight by age two [64], SGA mice did not show complete catchup growth by adulthood (or by weaning). This may be a consequence of their smaller size at birth, thus less success in competing for suckling than non-SGA littermates.

PCom Collaterals Evidence Large Stochastic Variation

The absence of a contribution of SGA to variation in PComs among mice of the same age, genetic background (B6), and postnatal environment suggests that stochastic (random) developmental variation among littermates is a major contributor to the large variation in PCom number in BALB/c and B6

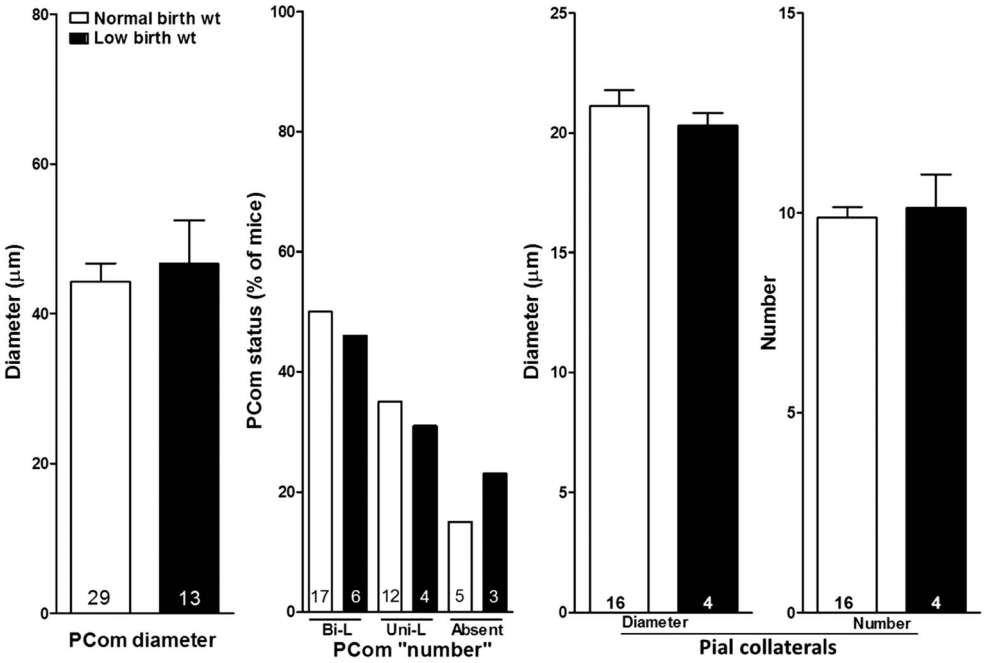

Fig. 4 PCom and pial collaterals are not altered in mice born small for gestational age (SGA). Low birth weight C57BL/6 mice (i.e., lower 12.5 percentile of newborn weight) evidenced catchup growth of brain weight by adulthood (10 weeks of age) but not of body weight by the time of weaning or adulthood (Supplemental Table II). The PCom diameter did not differ in female versus male mice of normal (70 ± 20 μm, $n = 4$, 60 ± 14, $n = 11$) or low birth weight (33 ± 6, $n = 2$, 30 ± 3, $n = 4$). PCom absence was not significantly different in normal versus low birth weight mice (40 vs 50%) or for overall status (ND), nor was it between sexes (Supplemental Table II)

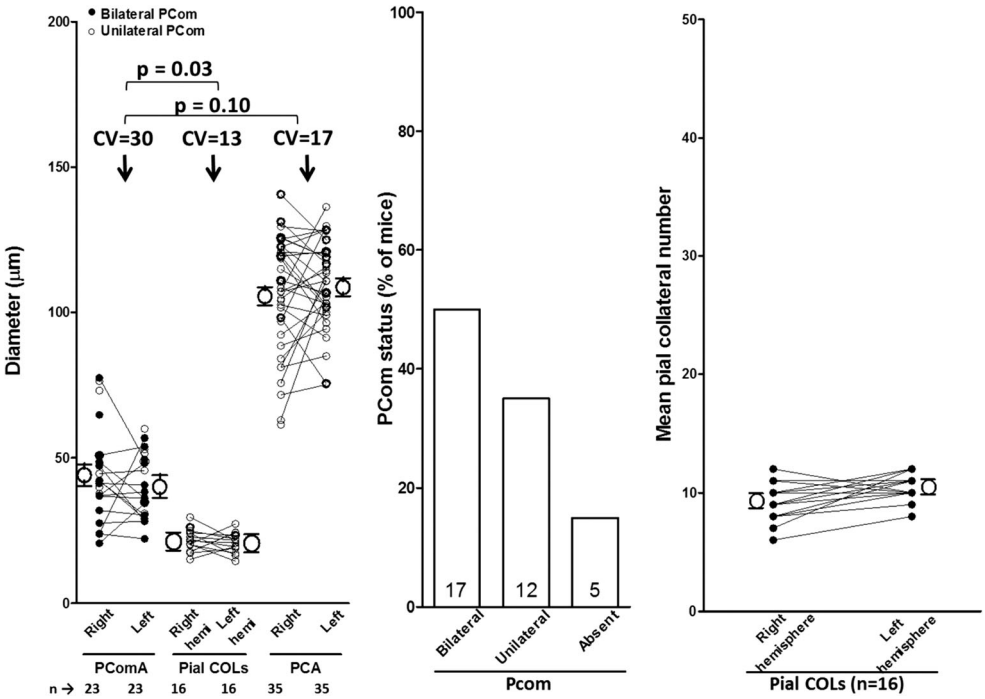

Fig. 5 PCom number and diameter evidence large stochastic variation compared to PCA and pial collaterals. Analysis was for male and female mice (no sex differences were observed) at 10 weeks of age that had normal birth weight in the intrauterine growth restriction experiment shown in Fig. 4. Lines that connect 2 dots represent the same individual. CV, coefficient of variation; *p* values by Modified Leven's test (Brown-Forsyth test), one-tailed

mice, as well as diameter of the right versus left PCom of the same individual in these and the other six strains. Support for this hypothesis is shown in Fig. 5, wherein only the non-SGA mice in the SGA study were examined to avoid potential pre- or postnatal effects of SGA. Variation in diameter of right versus left PComs was greater than variation in diameter of pial collaterals and PCAs. Likewise, variation in PCom number was many fold higher than for PCAs and pial collaterals (note that pial collaterals number was comparable in right and left hemispheres). Similar to the findings for the strains in Fig. 1 (data not shown), the mean diameter did not differ for left versus right sidedness among the PComs, PCAs, and pial collaterals. And the difference in diameter for the right and left PComs did not correlate with a directionally same difference in PCA diameter. This suggests, like the preceding results, that differences in the PCom diameter are not simply secondary to differences in PCA diameter or visa versa.

MCA Occlusion Distal to the Circle of Willis Induces Outward Remodeling of PComs

Increased blood flow induces outward remodeling (anatomic lumen enlargement) of arteries, arterioles, and collaterals [58], including PComs after carotid occlusion [48]. Although it is commonly assumed that flow across the PComs is not or minimally recruited when the site of occlusion is distal to the CoW, e.g. [72–74], we are unaware of a study examining this question. Figure 6 shows that this assumption is incorrect. Outward remodeling of PComs was assessed 6 days after pMCAO in five strains (only B6 and BALB/c mice with bilateral PComs were evaluated). Remodeling showed large strain-specific differences and occurred in the ipsilesional PCom in all strains and also in the contralesional PCom in three strains. The amount of remodeling did not associate with baseline diameter (or length) of PComs, PCAs, PCA or MCA territories, or pial collateral extent (Supplemental Table I). In contrast to PComs, we previously found that remodeling of ipsilesional pial collaterals examined 6 days after pMCAO for the five strains in Fig. 6 did not differ (2 to 4 fold increase in diameter; contralesional pial collaterals were unaffected) [50].

Discussion

The main findings of our study are, first, that wide variation was identified in the PCom diameter and number among six strains of inbred mice, indicating that naturally occurring differences in genetic background can be a major cause of variation in PComs. This conclusion was underscored in an F1 hybrid wherein the 129Sv genome exerted dominance for its robust PCom phenotype over the B6's phenotype of small diameter and highly variable number. Second, deletion of *Rabep2*, whose variant alleles are primary determinants of differences in embryonic formation of pial collateral arterioles, and thus, a major cause of the wide variation in pial collateral extent in the adult [52, 53], had no effect on extent of PCom collateral arteries. This suggests that different polymorphic genes and signaling pathways are involved in the formation of PCom versus pial collaterals. Third, random stochastic factors are also a strong contributor to variation in

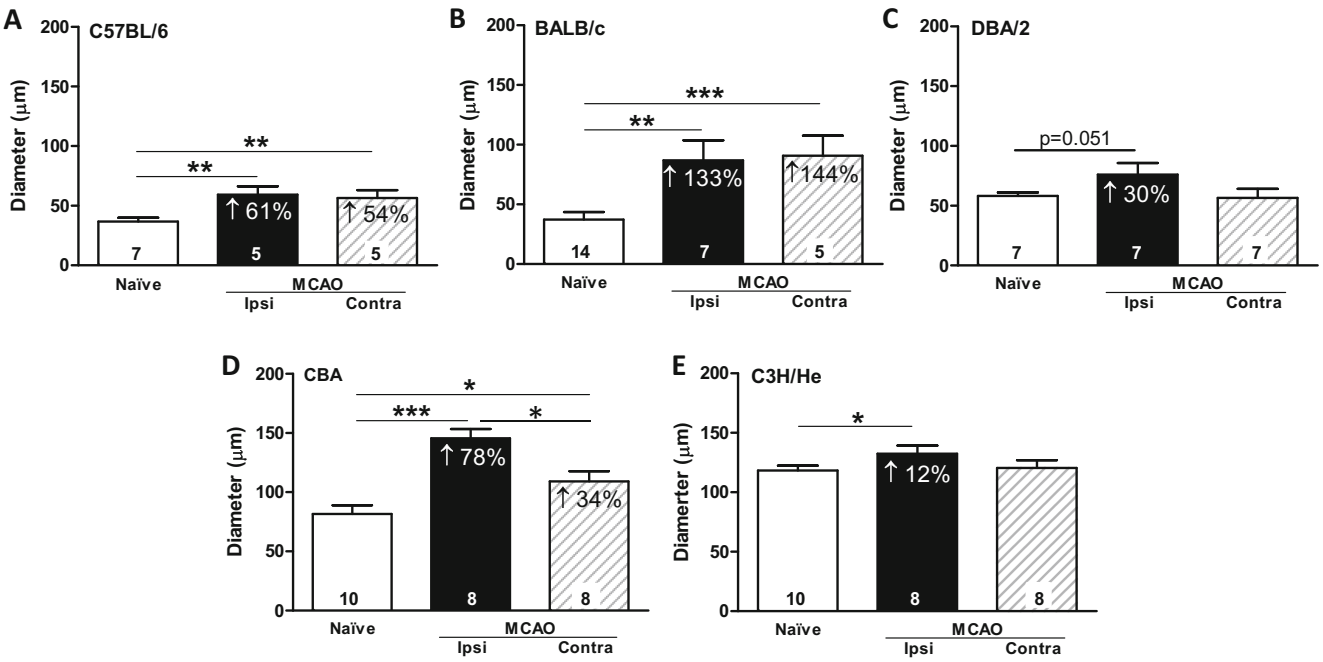

Fig. 6 **a**–**e** MCA occlusion distal to the circle of Willis induces outward remodeling of both ipsilesional and contralesional PComs that varies with genetic background. Remodeling, assessed 6 days after MCAO, was greater for PComs ipsilateral to MCAO in 4/5 strains and was the only side to remodel in DBA/2 and C3H/He. (**a**-**e**) The pattern of strain-specific variation in remodeling of PComs differs from the pattern of variation in remodeling of pial collaterals in the same strains [49], suggesting differences in the pathways that govern their remodeling. *, **, ***, $p < 0.05$, 0.01, 0.001, 3-month-old male mice. Values for naïve (no MCAO) were confined to mice with uni- or bilateral PComs. Data after MCAO were obtained from mice with bilateral PComs. Variation in remodeling did not correlate with baseline (naïve) diameter

PCom (but not pial) collaterals. Fourth, environmental factors affect PComs but with less impact than genetic or stochastic factors: age and hypertension reduced PCom diameter; however, obesity, hyperlipidemia, metabolic syndrome, diabetes mellitus, and intrauterine growth restriction had no effect. Fifth, unilateral pMCAO caused outward remodeling of both ipsilesional and contralesional PComs that varied with genetic background, indicating that occlusion distal to the CoW recruits flow across the PComs.

Correlation analysis among the above strains showed that variation in the PCom diameter was not secondary to differences in brain weight and was only weakly associated, directly, with variation in PCA diameter and indirectly with PCA territory. Two previous studies have identified an effect of gene deletion on ACom status [25, 75]. In B6 embryos with targeted smooth muscle cell deletion of RBP_j (that codes for a transcription factor required for downstream notch signaling), AComs were enlarged and had reduced SMC content, and M1-MCAs and A1-ACAs were tortuous, had non-uniform calibers and were hypoplastic or absent unilaterally [25]. We confirmed these findings in 4-week-old 129Sv;B6 SM22-$Cre^{+/-}RBPj^{fl/fl}$ mice versus their *Cre*-negative littermates (Lucitti and Faber, unpublished; pial collateral number was not different). Luna et al. [75] found that ACom absence was more frequent in B6 mice with deletion of placental growth factor; however, this may arise from the absence, duplication, or hypoplasia of the ICAs that were also noted. PComs were not evaluated in the above studies. Also, the above studies employed gene knockout and thus do not address whether naturally occurring polymorphisms of the above genes contribute to ACom variation in mice, or whether the ACom variation is secondary to the large disturbances that also occur in formation of the primary intracerebral arteries.

Hecht and coworkers [48] reported number and outside diameter of PComs of B6 mice that agree closely with our values. Unlike PComs, AComs were uniformly present and of large diameter [48], a difference that may arise because the PComs and other vessels of the posterior CoW begin to develop first, followed by vessels of the anterior CoW and AComs [3, 21–29]. Interestingly, pial collaterals form last [54, 76, 77]. As well, the PComs and AComs are collateral arteries, whereas pial collaterals are arterioles that cross-connect the crowns of adjacent arterial trees. The above considerations are congruent with the data in Figs. 1 and 2 indicating that different genetic variants are involved in formation of PCom versus pial collaterals. They are also consistent with previous reports that the 2 to 3 large diameter artery-like collaterals in the rectus abdominus [57] and gracilis muscles [78] do not differ in number between B6 and BALB/c mice (and at least in the gracilis are present as large anastomotic arteries in the neonate [79]), yet the number of small collaterals within the microcirculation of these same and other muscles (and in

the intestine and brain) differ greatly between the two strains [57, 78]. Thus, the genetic polymorphisms that govern variation in macro- and microvascular collaterals appear to differ.

Earlier reports employing India ink angiography suggested that PCom variation exists among different strains of mice; however, significant disagreement exists among the reports (discussed below). As well, comparisons with our findings are complicated since previous studies did not use perfusion fixation at maximal dilation, often lacked quantification, relied on smaller *n* sizes, and in several studies [38–41] did not differentiate among unilateral, bilateral, or absent PComs. Thus, PComs were reportedly "intact" (bilateral) in 10 each of B6/J, BALB/cCF, and 129/J mice [42], lacked "gross differences" in B6 and 129/J [43], and were "bilateral and hypoplastic" (small diameter) in B6/Tac versus "well formed" in 129Sv/Tac [44]. The above findings are at variance with our results. Barone et al. [45] reported that PComs in BALB/cCr were unilaterally present in 56%, absent in 44%, and bilateral in none—findings that agree somewhat with our data. Wellons et al. [46] obtained PCom diameters for B6/NTac and 129SvEv/Tac that were similar to our findings; however, the PCom number was not reported. Despite differences in methodology, substrains, and lack of agreement, the above studies when taken together suggest that genetic-dependent variation in PComs may exist among mouse strains—a suggestion that our results confirm. Knowledge about the cause of variation in extent of PComs is important not only for basic understanding but also because variation impacts the risk and severity of cerebral ischemia and intracranial aneurisms in humans [3–5, 12, 31–38, 80, 81] and in animal models of stroke employing obstruction of arteries proximal to and within the CoW. The latter include the filament model for temporary or pMCAO. Insertion of the filament recruits flow through extant PComs during ligation of the ICA. In addition, thrombus formation at the ICA-MCA junction could illicit embolic obstruction of the ipsilesional PCom after withdrawal of the filament. It is well known that the filament model is accompanied by significant variation in ischemia and infarction, including in the same mouse strain [39–41, 43–47, 49, 82, 83] that some investigators have attributed to variation in CoW collaterals. Moreover, the B6 strain, which had the greatest within-strain variation in PComs in our study, is commonly used to create targeted mutations. Our findings thus clarify potential sources of disagreement in previous studies of PCom variation and infarct volume in mouse models of stroke (e.g., see Supplemental Figure IV).

Similar to mice (and other mammals [1, 49, 84]), variation in the collaterals of the CoW is also prominent in humans [2–24, 28–38]. The following are averages that we calculated from values reported in cadaveric studies (which unlike radiologic studies are not limited by imaging resolution) that examined at least 100 adult humans of varying ethnicities without known cerebral disease: 28% had a complete CoW with no hypoplastic vessels, generally defined as diameter of < 1 mm for CoW arteries, < 0.5 mm for PCom or ACom (the most frequently used cutoffs [3]) and no absent PComs or ACom [6, 15–20]. In the remainder, the most common variants were PComs that were bilaterally hypoplastic (28% [15–19]), unilaterally hypoplastic (18% [15–19]) or absent (1% [6, 20]). Variation in the CoW has also been reported in angiographic studies. In the largest study to date (834 CTAs), Zaninovich and coworkers [7] found that the incidence of a complete CoW was 38% when averaged across age groups. Bilateral absence (of detection) of PComs averaged 17% (vessel diameters were not measured). The absence of the right PCom, the second-most common variation, averaged 16%. These variants were more common in males. The absence of ACom and P1-PCAs each averaged 3%. These values approximate the values in the cadaveric studies, although they differ somewhat, most likely from lower resolution. Other angiographic studies have reported variation in the pattern of the CoW, including vessel diameters. A "fetal origin of the PCA" (i.e., the rodent P1-PCA configuration) was present unilaterally in ~ 14% and bilaterally in 0.5%, while a hypoplastic P1-PCA was present in up to 10% [3]. Unilateral or bilateral absence of PComs was evident in 6%. Krabbe-Hartkamp et al. [9] reported a patent complete CoW in ~ 25% of patients. Van Raamt and coworkers [4] reported the presence of fetal and adult patterns in 25 and 75%, respectively. In healthy individuals, Li et al. [10] found 69% had incomplete PComs and 9% had at least one fetal PCA; Macchi and colleagues [8] reported incomplete CoWs in 59%, hypoplastic PComs in 21%, fetal PCAs with one or both P1-PCAs detectable in 13%, and the latter not detectable in 2%; Qui et al. [13] examined 2246 males of 50 years average age and reported that 48% had an adult PCA pattern and non-detectable PComs, 23% had one PCom, 15% had hypoplastic PComs on one or both sides, 5% had the fetal PCA pattern, and 1% had no detectable AComs. Similar findings have been reported by others [11, 14].

Formation of the CoW, which involves sequential cooption and remodeling of segments of the transient aortic arches, occurs from gestational day 11.5 to 15.5 in mice [24–27] and day 40 to 55 in humans [3, 4, 21–23, 28, 29]. Since variant patterns of the CoW are established before normal term, incomplete CoW variants are referred to as congenital. Formation of the posterior components is especially variable (discussed below). Hypoplasia or absence of one or more of the PComs or ACom, which become evident by 4 to 7 weeks and attain their full diameters by 16-week gestation, has been postulated to result from deficient retension of early embryonic arteries [3, 24]. However, potential cause(s) of this have not been investigated. We hypothesize that differences among individuals in genetic and stochastic factors during development contribute to PCom variation, either directly through effects on vascular cell function and/or secondarily by affecting the rate of growth of the telencephalon versus

diencephalon. And that certain environmental factors evident after birth such as aging and hypertension can further affect PCom extent.

We are unaware of studies investigating whether genetic factors contribute to variation in the CoW in humans, other than a single case report finding apparent familial linkage [85]. However, studies of different ethnicities are consistent with this possibility. The following summarizes findings of cadaveric studies that examined 100 to 1000 adults lacking known cerebral disease; the percent incidence of a complete CoW with no hypoplastic vessels is as follows: 14% Sri Lanka [15], 15% France [16], 18% Morroco [17], 22% Serbia [18], 28% Iran [19], 45% India [20], and 52% US [6]. Five of these studies reported the incidence of bilateral PCom hypoplasia as follows (%): 23 [15], 24 [17], 25 [18], 27 [19], and 42 [16]; and unilateral PCom hypoplasia as follows (%): 12 [15], 14 [17], 14 [16], 20 [19], and 32 [18] while two of the studies both reported absence of PCom in 1% (note that denoting vessels as hypoplastic in the above studies does not differentiate between patent versus non-patent vessels) [6, 20]. The contributions of genetic, non-genetic, and technical factors to the above differences are not known.

The incidence and severity of cerebral ischemic diseases increase with age. While many factors are involved, we previously found that pial collateral number and diameter decline progressively in young adult, middle, and advanced-age B6 mice (3, 16, 24–31 months of age), in association with increased infarct volume following pMCAO [55, 58]. In the present study, diameter of PComs in B6 mice also declined over a similar time-course. We are unaware of other animal studies examining PComs (or AComs) and aging. In humans, Zaninovich and coworkers [7] reported that the incidence of a complete CoW on CTA, which was 52% in 18–39 year olds, declined with middle (40–69 years, 36%), and advanced age (≥ 70 years, 25%). The bilateral absence of PComs in young individuals (10%) increased with middle and advanced age (19 and 21%, respectively). The absence of the right PCom also increased with age (13, 15, and 19%). The absence of ACom and P1-PCAs (each 3% overall) did not change with age. These findings agree with previous studies [11, 63], although not entirely [9, 14] and likely reflect at least in part a decline in PCom diameter below the resolution of CTA imaging. They also agree with our finding of decreased PCom diameter with aging in B6 mice. However, PCom diameter in B6;129Sv mice increased over 3, 8, and 12 months of age. This may reflect normal growth of PComs to the larger diameter evident on reaching full adulthood. Although B6;129Sv mice may evidence, like B6, a decline in diameter with additional aging (and B6 may show an increase at early ages similar to this mixed strain), these findings indicate that the effects of age vary with genetic background. Zaninovich et al. [7] suggested that PComs may "occlude over time from atherosclerosis and other diseases", which is supported by Chuang et al. [12] who found that hypoplastic PComs were more common in patients with small vessel disease but not with other TOAST stroke phenotypes. In support, we found that hypertension reduced PCom diameter; however, hyperlipidemia, metabolic syndrome, obesity, or diabetes mellitus were without effect (discussed below).

In contrast to PCom diameter, PCA diameter did not decline but instead increased through middle age in both B6 and B6;129Sv mice. This is consistent with our previous study wherein PCA, MCA, ACA, ICA, and BA trended or significantly increased in 26 versus 3-month-old B6 mice [58]. Outward remodeling of carotids has been observed with aging, whereas conflicting reports exist for the primary intracranial arteries, possibly due to variable presence of CSRFs or differences in resolution of CTA and MRA [9, see also 58]. For example, Shatri et al. [63] analyzed MRAs of 133 individuals—equally divided for sex and age (< 40 years, ≥ 40 years) and lacking known cerebral disease or arterial or venous malformations. Diameters of PComs, ICAs, and BAs were smaller in the older group while PCAs, AComs, and the other intracerebral arteries did not differ. It has been suggested that an effect of age to promote outward remodeling or lessen an age-associated decline in diameter of the PCA and other cerebral arteries could compensate for the adverse effects of age and CSRF presence on microvascular dimensions and autoregulatory capacity and thus ameliorate the decline in cerebral blood flow that occurs with aging [58].

The increase in diameter of the PComs and PCAs in middle-age B6;129Sv mice was reduced and abolished, respectively, by hypertension. This is somewhat in line with the reduction in number and diameter (rarefaction) of pial collaterals, which increased with the severity and duration of hypertension, that we reported previously in the same strain [57]. Likewise, hypertension has been associated with smaller lumen diameter and increased wall-to-lumen ratio in cerebral arteries and arterioles in animals [86, 87] and humans [88]. Warnert and colleagues [61] found that individuals with an incomplete CoW on MRI (i.e., hypoplastic PComs or uni- and/or bilateral absence of detection; no cerebral stenotic disease) were 60% more likely to have hypertension (odds ratio of 2.6), while no association was seen in those with undetectable or absent AComs. Similar findings have been reported by others [12, 62].

We previously found that obesity, metabolic syndrome, hyperlipidemia, and diabetes mellitus cause rarefaction of pial collaterals [57]. In the present study, these CSRFs had no effect on PCom diameter (or PCA diameter). Similarly, diameters of the PCom and the primary intracerebral arteries were not different in 4–5-month-old obese mice, except that PCA was 10% smaller ($P < 0.05$) [89]. We are unaware of other animal or human studies examining whether CSRFs contribute to variation of the CoW. Mechanisms underlying anatomic changes in PComs, pial collaterals, and intracranial arteries with aging and hypertension may involve the decline in

eNOS bioavailability that is well known to occur in both conditions, [see 58]. Indeed, genetic increase in eNOS or exercise training prevented age-induced rarefaction of PComs and pial collaterals and promoted outward remodeling of the primary intracranial arteries [58].

PCom number in B6 and BALB/c mice evidenced high within-strain variability even though mice of each strain were isogenic, the same age, and conceived and raised in the same vivarium. PCom diameter on a given side among individuals, and for right versus left side of the same individual, also varied widely within these and the other strains in Fig. 1. These findings, which suggest that inter-individual differences in non-genetic factors during development strongly contribute to variation in PComs, led us to hypothesize that PCom insufficiency occurs in individuals born with low birth weight ("runts"), resulting in the above variability in the same strain. When not due to preterm delivery, low birth weight (≤ 10%) is termed intrauterine growth restriction or small for gestational age (SGA) [64]. SGA has been associated with a variety of maternal risk factors, including young age, poor nutrition, heart disease, hypertension, diabetes, anemia, hypoxemia, drug or alcohol abuse, insufficient prenatal care, poverty, smoking, air pollutants, heavy metal exposure, and utero-placental problems. When caused by a chromosomal or genetic mutation, it is termed "constitutional" SGA. The appearance of runts within litter-bearing species is a model of naturally occurring SGA [70, 71]. SGA has been linked to increased infant, childhood and adult morbidity and mortality, decreased brain and body growth and cognitive development, and hypertension, dyslipidemia, insulin resistance, and other diseases in adulthood. The 8–10% incidence of SGA in developed countries is higher and increasing in developing countries due to better preterm survival. Of particular relevance to our abovementioned hypothesis, the incidences of ischemic stroke and poor outcome have been associated with SGA, irrespective of sex or hypertension [64–67]. Notwithstanding the above rationales, we found that the PCom diameter and number were comparable for SGA and non-SGA B6 littermates. Interestingly, De Felice et al. [90] found that 100% of SGA children having a congenital variation in auricular shape (which was also present in their mothers but not fathers) had bilaterally reduced or absent PComs on transcranial Doppler during common carotid compression, compared to 0 % in controls. The affected children also had subclinical cochlear dysfunction. The authors hypothesized that the association between the ear and PCom phenotypes results from a genetic-dependent aberrant development of the pharyngeal arch mesoderm. The apparent linked-genetic basis for these phenotypic variations is consistent with our findings of a strong genetic contribution to PCom variation among different mouse strains, yet no contribution of SGA in mice where genetic differences have been excluded.

The absence of contribution of SGA suggests that "stochastic" (random) developmental variation among the littermates is a major contributor to the variation in PCom number that is especially evident in the C57BL/6 and BALB/c backgrounds. We tested and affirmed this hypothesis by analyzing the non-SGA B6 mice to avoid any confounding effect of unknown SGA-associated environmental factors that might be present during gestation. For example, lower blood flow in embryos located furthest from uterine arterial inflow is believed to result in SGA in litter-bearing species [70, 71]. Interestingly, stochastic variation may also contribute to the well-known variation in human collateral arteries in the CoW, limbs, and other tissues. For example, ~ 40% of subjects examined had complete and ~ 60% had sparse or absent palmar arch collateral arteries [91], and Cambron and coworkers showed that complete arches can be present in the one hand but not the other hand of the same individual [68]. The fact that the CoW is incomplete in a large proportion of humans, as reviewed above, and in mice as shown in our study (and presumably in other species) suggests that the physiological role of the CoW postulated by Vrselja et al. [1] if correct, provides minimal advantage for reproductive fitness since the phenotype varies so widely in these species.

Sustained increase in flow causes shear stress-induced outward remodeling of arteries, arterioles, and collaterals in various tissues, including pial collaterals in the brain [see 58]. PComs undergo remodeling after bilateral carotid occlusion [48, 92; however, see 93]. Remodeling of both collateral types in the brain aids recovery of vasodilator reserve [92, 94]. For example, 1 week after ligation of one carotid and both vertebral arteries in a rat, the ipsilateral PCom, ACA, and MCA remodeled by 39, 16, and 10%, respectively, in association with recovery of cerebrovascular dilation to carbon dioxide [92]. Similar results have been reported for B6 mice [48]. It is commonly assumed that flow across the PComs, which is normally low or absent due to lack of a pressure difference [1, 95, 96], is not or minimally recruited when the site of occlusion is distal to the CoW [72–74]. However, no studies have examined this question. In contrast to the above assumption, ipsilesional and contralesional PComs remodeled by similar amounts, averaging 56, 58, and 139% for both sides combined, in three of five strains that we examined 6 days after unilateral pMCAO. In the other strains, less remodeling occurred and only of the ipsilesional PCom (12 and 30%). Remodeling of pial collaterals among the above strains varies from 2 to 4 fold, but the differences in remodeling of PCom and pial collaterals among the strains are not concordant [50]. Furthermore, the percentage amounts of remodeling of PCom and pial collaterals among the strains do not correlate with differences in their baseline values for number, diameter or length of the PCom and pial collaterals, territories of the MCA and PCA, arterial pressure, or hematocrit—factors that affect the magnitude of the shear stress stimulus [50]. Thus, the absence of contralesional PCom remodeling in the two strains may reflect a genetic-dependent difference in activity of signaling within the remodeling pathway. Pial collateral remodeling did not

show a deficit in these strains [50], which suggests that the pathways governing remodeling of collateral arteries and collateral arterioles differ. Interestingly, the rank order for the amount of remodeling we observed for PCom arteries agrees with remodeling of the right carotid artery measured 2 weeks after stenosis of the left carotid, where B6 > DBA/2 > C3H/He (BALB/c and CBA were not examined) [97]. Greater remodeling of the ipsilesional PCom that was evident in four of five strains is consistent with the expectation that MCAO induces retrograde flow across the ipsilesional PCA-to-MCA pial collaterals that is then supported by flow diversion across the ipsilesional PCom together with contribution from the contralesional PCom and BA. Such a mechanism may contribute to the recent findings of van Seeters and colleagues [98].

This study has limitations. Given the high variability in PCom number, sample sizes were insufficient to test for an effect of sex or CSRFs. It is possible that a longer presence or later time of onset of a given CSRF may yield different findings. The genetic mouse models of obesity, hyperlipidemia, metabolic syndrome, diabetes mellitus, and hypertension that we studied may not mimic these diseases in humans, for example, the obesity model did not consist of feeding wildtype mice a high caloric diet. Other potential inter-individual differences in non-genetic and non-SGA environmental or epigenetic factors arising in utero may contribute to what we denote as the "stochastic" component of variation in PCom extent. Given that the effects of aging, stochastic events, and remodeling of PComs varied with genetic background, our conclusions about the contribution of these factors cannot be generalized to all mouse strains or the mouse as a species.

In conclusion, our findings show that genetic and stochastic factors have significantly stronger effects than environmental factors (i.e., CSRFs) on variation in the extent of PComs. They also demonstrate that flow across both PComs is recruited by occlusion distal to the CoW. Thus, besides their well-known contribution to stroke severity after obstruction of the carotid, vertebral, or BAs, variation in the extent of the PComs at baseline and their remodeling after obstruction may also contribute to variation in tissue injury and outcome in patients with obstruction distal to the CoW. Interestingly, an incomplete ipsilesional posterior CoW in patients with MCA occlusion was independently related to poor leptomeningeal collateral flow (OR 1.7; 95% CI 1.1–2.6) [98]. Our results also indicate that variation in the extent of PCom collateral arteries and pial collateral arterioles at baseline arise from different genetic polymorphisms, suggesting that different mechanisms determine their formation during gestation. We also find that aging and other CSRFs and stochastic factors affect the two collateral types differently. Finally, our study clarifies uncertainties arising from previous studies of PCom variation and infarct volume in mouse models of ischemic stroke and identifies mechanisms that may underlie PCom variation in humans.

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Author Contributions JF designed the study and figures and wrote the manuscript. HZ performed angiography, pMCAO, pial collateral morphometry, statistical analysis, animal husbandry, and finalized the figures. WR, KD, BS, CB, and SH performed the morphometry of PComs and primary cerebral arteries and statistical analysis.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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