Basic Sciences

Cerebral Hemodynamic Reserve and Vascular Remodeling in C57/BL6 Mice Are Influenced by Age

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Background and Purpose—Age is the most important risk factor for ischemic stroke. Recent experiments evidenced an age-associated rarefaction of the native collateral vasculature. The purpose of this study was to assess in what way age and arteriogenesis influence cortical perfusion and recovery of hemodynamic impairment in aged and young C57/BL6 mice.

Methods—After model establishment of chronic cerebral hypoperfusion in the C57/BL6 strain, sustained hemodynamic impairment was induced by permanent unilateral internal carotid artery occlusion in animals aged 4 to 6 weeks, 12 weeks, and 18 months. Functional and morphological outcome was assessed by laser speckle imaging before and during acetazolamide challenge on Days 0, 3, 7, and 14 and latex/carbon black angiography and immunohistochemistry on Day 21.

Results—Although internal carotid artery occlusion did not result in a reduction of baseline perfusion, it led to significant hemodynamic impairment in all age groups. Furthermore, baseline perfusion in sham and cerebrovascular reactivity after internal carotid artery occlusion were significantly lower in animals aged 18 months (468±57 Flux; 20.8%±17%) compared with mice aged 4 to 6 weeks (568±120 Flux; 30.3%±17%) and 12 weeks (591±72 Flux; 34.2%±12%) from the beginning until Day 7 of the monitoring period. Functional outcome was in line with a 27% reduction of native leptomeningeal anastomoses in aged mice and only limited collateral outgrowth compared with young animals. Strikingly, all age groups reached spontaneous functional compensation by Day 14.

Conclusions—Next to limited collateral remodeling, our results suggest that a hampered cerebrovascular response with age could intensify the risk for hemodynamic stroke in the elderly. (Stroke. 2012;43:3052-3062.)

Key Words: aging ■ arteriogenesis ■ cerebral blood flow ■ cerebrovascular reserve capacity ■ collateral circulation ■ laser speckle imaging

Age has been identified as the most important risk factor for ischemic stroke and 10% of these cases are due to hemodynamic compromise. The major cause of hemodynamic compromise is symptomatic internal carotid artery occlusion with an annual ischemic stroke risk of 5.5% to 10%. This risk correlates with the degree of hemodynamic impairment and may reach up to 41% per year in patients with an exhausted cerebrovascular reserve capacity (CVRC). The most important rescue mechanisms to cope with this chronic hypopemia are the natural outgrowth of pre-existing collaterals, that is, arteriogenesis and regulation of blood flow through an intact endothelial function. Experimental findings suggest that the natural capacity for arteriogenesis may be limited in aged patients in conjunction with a functional impairment of the endothelial monolayer.

Natural or stimulated cerebrovascular remodeling has previously been demonstrated in a rat model of cerebral hypoperfusion. A recent study in C57/BL6 mice nicely demonstrated that aging causes rarefaction of the native leptomeningeal collateral vasculature and leads to a more severe ischemic tissue injury. However, in the setting of chronic cerebral hypoperfusion, the effect of age on the degree of collateral remodeling and functional recovery of the CVRC remains unclear.

Here, we developed a model of chronic cerebral hypoperfusion in the C57/BL6 mouse to investigate the influence of age on functional hemodynamic impairment and outward remodeling of pre-existing collaterals. Because translation of the classic “cerebral hypoperfusion rat model” to the mouse may be hampered by the excellent cerebrovascular collateralization and the variable interconnection between the anterior and posterior circulation through the P1 segments of the posterior cerebral artery (PCA) in the C57/BL6 strain, our experiments were performed in a 2-step approach. First,
we characterized the influence of the P1 segment distribution on perfusion in the anterior circulation after proximal vessel occlusion and investigated the morphological effect of chronic cerebral hypoperfusion in C57/BL6 mice aged 4 to 6 weeks. Second, hemodynamic impairment was investigated in C57/BL6 mice aged 4 to 6 weeks, 12 weeks, and 18 months and collateral remodeling was compared between the 4- to 6-week- and 18-month-old animals.

Methods

Ethics Statement
Experiments were permitted by the local ethics committee on animal research (LaGeSo No. G 0262/07, Berlin, Germany) and in conformity with the German Law for Animal Protection and the National Institute of Health Guidelines for Care and Use of Laboratory Animals.

Animals and Experimental Design
One hundred fourteen male C57/BL6 mice (Charles River WIGA GmbH, Sulzfeld, Germany) aged 4 to 6 weeks (23–27 g), 12 weeks (25–31 g), and 18 months (35–40 g) were used in this study and given free access to food and water before and after all procedures. Within each age group, animals were randomly assigned to 1 of 2 procedures: sham internal carotid artery (ICA) occlusion and right ICA occlusion. For all procedures, mice were anesthetized with 70 mg/kg ketamine and 16 mg/kg xylazine and body temperature was maintained at 37°C. Three series of experiments were performed: (1) The relevance of the P1 segment on collateral flow after mild hemodynamic impairment in the anterior circulation was estimated in mice aged 4 to 6 weeks; (2) to confirm arteriogenesis in the C57/BL6 mouse strain, the effect of permanent, unilateral (right) ICA occlusion on cortical vessel density, pericyte coverage, cell proliferation and basal vessel wall diameter was determined in mice aged 4 to 6 weeks; and (3) the effect of permanent, unilateral (right) ICA occlusion on the degree of hemodynamic impairment was determined in mice aged 4 to 6 weeks, 12 weeks, and 18 months and collateral vessel growth was assessed in the 4- to 6-week- and 18-month-old animals.

Measurement of Cerebral Perfusion Changes After Proximal Vessel Occlusion in Dependence of the P1 Segment Distribution
For the purpose of developing a reproducible model of nonlethal hypoperfusion, we characterized the influence of the P1 segment anatomy on collateral blood flow after uni- and bilateral ICA occlusion. For a detailed description, see the online-only Data Supplement.

Permanent ICA Occlusion
Permanent unilateral (right) ICA occlusion and sham procedures were performed in 60 C57/BL6 mice aged 4 to 6 weeks, 12 weeks, and 18 months (n=20 per group). After anesthesia, the right ICA was exposed by a midline neck incision, dissected from the vagal nerve and surrounding sheath, and ligated with a silk 8/0 suture. Sham procedures were performed in the same fashion without ligation. The skin was sutured with 6/0 nylon.

Measurement of Baseline Perfusion and CVRC by Laser Speckle Contrast Analysis
For a detailed description, see the online-only Data Supplement. Laser Speckle Contrast Analysis after permanent ICA occlusion and sham ICA occlusion was performed 30 minutes after surgery (Day 0) and on Days 3, 7, and 14 in animals aged 4 to 6 weeks, 12 weeks, and 18 months. The Laser Speckle Contrast Analysis imager (Moor Instruments, Devon, UK) was connected to a standard laptop computer equipped with purpose-designed data acquisition software (MoorFLPI Measurement Software Version 3.0; Moor Instruments).

Video frame rates of relative flow within the microcirculation were processed to a color-coded cerebral blood flow (CBF) Flux image that correlates with the blood flow velocity in the tissue (red=high flow, blue=low flow). In the present study, an image-sampling rate of 0.25 Hz with an exposure time of 4 ms was selected for all measurements. To minimize hemodynamic alterations such as cardiovascular and respiratory side effects that may hamper CVRC assessment and occur with volatile anesthetics, opioids, or benzodiazepines, we selected ketamine and xylazine as an anesthetic agent based on preliminary experiments.

Mice were placed in prone position and the skull was exposed by a midline scalp incision (2 cm). All CBF Flux measurements were obtained within a 6×4 mm region of interest over the right middle cerebral artery (MCA) territory, which permitted a combined arterial, venous, and parenchymal relative cortical perfusion assessment in the arbitrary unit Flux over the MCA territory ipsilateral to the site of ICA occlusion. A 2-minute baseline CBF Flux was recorded and a 60-second CBF Flux plateau was calculated for baseline perfusion analysis. Next, 50 mg/kg acetazolamide (Diamox; Goldshield Pharmaceuticals Ltd, Surrey, UK) was injected and the acetazolamide-specific CVRC was calculated as the percent increase between baseline and a 60-second CBF Flux plateau after a maximum rise in CBF. After imaging, the scalp was sutured with 6/0 nylon.

Assessment of the Cerebral Angloarchitecture by Latex/Carbon Black Perfusion
For a detailed description, see the online-only Data Supplement. Latex/carbon black perfusion in animals aged 4 to 6 weeks and 18 months was performed as described previously. Measurements of the diameter and number of leptomeningeal anastomoses of both hemispheres and the vessel diameters of the circle of Willis (anterior cerebral artery [ACA], MCA, ICA, P1 segment, posterior communicating artery, and basilar artery) were performed using free software image analysis software (ImageJ64, http://rsweb.nih.gov/ij/) as reported previously. To compare the diameters of leptomeningeal anastomoses, relative frequency histograms of vessel diameters were compiled for both hemispheres in each group.

Immunohistochemistry
For a detailed description, see the online-only Data Supplement. For analysis of cortical vessel density and pericyte coverage, a combined CD31/desmin/antismooth muscle actin (SMA) stain was performed. A group of mice aged 4 to 6 weeks was anesthetized and euthanized on Day 21 after sham or ICA occlusion (n=5 per group). Four-micron coronal cryosections of nonperfused brains were obtained from the bregma level +1.0 mm to −2.0 mm in 1.0-mm intervals. Vessel density was calculated as vessels/mm2. CD31/desmin and CD31/anti-SMA double-positive vessels were quantified by counting of CD31-positive vessels with desmin or anti-SMA colocalization, respectively. To compare the diameters of parenchymal CD31/anti-SMA double-positive vessels, we compiled a frequency distribution of vessel diameters in the MCA territory of both hemispheres. For purposes of calculating the desmin coverage of CD31-positive vessels, values of the CD31 vessel density for sham and ICA occlusion were set as 100% and relative values for desmin coverage calculated accordingly. For analysis of cell proliferation, a combined Ki67/CD31/anti-SMA stain was performed in a separate set of animals. C57/BL6 mice aged 4 to 6 weeks were anesthetized and euthanized on Days 1, 3, 7, and 21 after sham or ICA occlusion (n=3 per group and time point). Four-micron coronal cryosections of nonperfused brains were obtained at bregma levels corresponding to the basal vessel segments of the ACA (+0.0 mm), MCA (+0.0 mm), ICA (−1.0 mm), posterior communicating artery (−2.0 mm), and PCA (−3.0 mm). Distinct Ki67/CD31 or Ki67/anti-SMA colocalization was used to recognize proliferating endothelial and smooth muscle cells, respectively.
Statistical Analysis

Statistical tests are presented in the text. For a comprehensive description, see the online-only Data Supplement.

Results

Influence of the P1 Segment Anatomy on Cerebral Perfusion After Temporary Uni- and Bilateral ICA Occlusion in C57/BL6 Mice

Bilateral P1 segments were identified in 6 (29%), unilateral P1 segments in 9 (5 right-sided, 4 left-sided; 47%), and absent P1 segments in 5 (24%) animals (Figure 1A–C). The diameter of the P1 segment ranged from 35 μm to 105 μm with a median diameter of 59 μm. Unilateral ICA occlusion resulted in a CBF decrease to 82%±7% in the ipsilateral and 93%±7% in the contralateral MCA territory. No difference was found in the perfusion drop according to the P1 segment distribution and values are expressed as the percentage of the LDF flux decrease compared with baseline perfusion. For the purpose of establishing a model of nonischemic cerebral hypoperfusion in C57/BL7 mice, the anatomy of the P1 segment had no significant influence on the cortical perfusion drop after proximal ICAO. ICA indicates internal carotid artery; CBF, cerebral blood flow; LDF, Laser Doppler Flowmetry.

Assessment of Vascular Proliferation and Characterization of the Cortical Microvasculature After Unilateral ICA Occlusion in C57/BL6 Mice

After unilateral ICA occlusion, we found a marked proliferation of endothelial cells within the basal vessels of the anterior circulation of the circle of Willis. In sham-operated animals, no endothelial cell proliferation was detected (Figure 2A). Proliferating cells within the smooth muscle cell layer were noted in both groups but more frequently in animals with ICA occlusion. Overall, endothelial and smooth muscle cell proliferation was most pronounced at Day 7 after surgery. At this time point, outward remodeling was confirmed by a significant increase in the vessel wall diameter of the ACA and MCA segments compared with sham (online-only Data Supplement Figure I). Proliferating endothelial cells were also present in vessel segments of the posterior circulation; however, proliferation within the P1 segment of the PCA was only infrequently detected. Next, we characterized vessel density and maturity of the parenchymal microvasculature within the cortical MCA territory of both hemispheres. On Day 21 after unilateral ICA occlusion, the microvascular density increased significantly compared with nonischemic sham animals (Figure 2B; sham: 441.9±8.1/mm²; ICA occlusion: 472.3±11.1/mm²; Figure 2D: *P<0.05 sham versus
ICA occlusion; Student t test for “CD31”). In sham-operated animals, 63.5±1% of these vessels showed colocalization with desmin compared with a colocalization of 57±1% in animals with ICA occlusion (Figure 2D: ***P<0.001 sham versus ICA occlusion; Student t test for “desmin”). Frequency distribution analysis revealed a 21.2% increase in the total number of anti-SMA-positive vessels (Figure 2D; sham: 15±0.9.1/mm², ICA occlusion: 19±1.1/mm²) and an increase
of anti-SMA-positive vessels with diameters ranging from 6 to 10 μm (Figure 2C: *P<0.05 sham versus ICA occlusion; 2-way analysis of variance and Figure 2D: **P<0.01 sham versus ICA occlusion; Student t test for “anti-SMA”).

Baseline Perfusion After Permanent Unilateral (Right) ICA Occlusion in C57/BL6 Mice Aged 4 to 6 Weeks, 12 Weeks, and 18 Months

In all age groups, unilateral ICA occlusion did not lead to a reduction of baseline perfusion over the ipsilateral MCA territory. No difference was noted between the baseline perfusion of mice aged 4 to 6 and 12 weeks (Figure 3A). Animals aged 18 months, however, had a significantly lower baseline perfusion than mice aged 4 to 6 or 12 weeks over the course of the entire monitoring period (Figure 3A: *P<0.01 and Figure 3B: *P<0.05 12 weeks versus 18 months; #P<0.05, 4–6 weeks versus 18 months; 2-way analysis of variance for repeated measures).

CVRC After Permanent Unilateral (Right) ICA Occlusion in C57/BL6 Mice Aged 4 to 6 Weeks, 12 Weeks, and 18 Months

In all age groups, unilateral ICA occlusion led to a significant reduction of the hemodynamic response to acetazolamide. Furthermore, ICA occlusion in animals aged 18 months resulted in a significantly lower CVRC than ICA occlusion in mice aged 4 to 6 or 12 weeks, respectively (Figure 4C: *P<0.01; 2-way analysis of variance for repeated measures). Although the CVRC on Day 0 was already significantly lower in old mice compared with young animals (CVRC after ICA occlusion on Day 0: 18 months 6%±4%; 4–6 weeks 18.4%±11%; 12 weeks 24.3%±12%), functional compensation occurred in all groups by Day 14 (CVRC after ICA occlusion on Day 14: 18 months 41.3%±13%; 4–6 weeks 38.6%±21%; 12 weeks 42.4%±8%; Figure 4D: *P<0.05 “4–6 weeks” sham versus ICA occlusion; *P<0.001 “18 months” sham versus ICA occlusion; §P<0.01 “ICA occlusion” 18 months versus 12 weeks; #P<0.05 “ICA occlusion” 18 months versus 4–6 weeks; 2-way analysis of variance for repeated measures). No difference was found between the CVRC of animals aged 4 to 6 and 12 weeks or between nons ischemic sham animals of all age groups.

Collateral Vessel Growth on Day 21 After Unilateral (Right) ICA Occlusion in C57/BL6 Mice Aged 4 to 6 Weeks and 18 Months

Unilateral ICA occlusion in mice aged 4 to 6 weeks resulted in a significant increase of the external vessel diameter of the
ACA, MCA, ICA, posterior communicating artery, and basilar artery compared with nonischemic sham animals (Figure 5A). No change was noted in the P1 segment diameter. ICA occlusion in mice aged 18 months led to a significant diameter increase in the ACA segment (Figure 5B). No difference was noted between the external vessel diameters of nonischemic sham animals (Figure 5A–B: *P < 0.05, **P < 0.01, ***P < 0.001, sham versus ICA occlusion; one-way analysis of variance).

In mice aged 4 to 6 weeks, ICA occlusion led to a significant increase in the relative frequency of larger caliber leptomeningeal anastomoses (25–35 μm: sham 27%±13%, ICA occlusion 40%±13%) that coincided with a reduction of smaller caliber anastomoses (15–25 μm: sham 49%±11%, ICA occlusion 36%±13%; 5–15 μm: sham 19%±9%, ICA occlusion 8%±4%; Figure 6A: **P<0.01, ***P<0.001, sham versus ICA occlusion; 2-way analysis of variance). In animals aged 18 months, no difference in the diameter of the leptomeningeal anastomoses was detected (Figure 6B). In both age groups, unilateral ICA occlusion did not result in a significant difference of the total number of leptomeningeal anastomoses (Figure 6A–B). However, we detected a 27% higher number of native leptomeningeal anastomoses in sham animals aged 4 to 6 weeks (28±5) compared with sham animals aged 18 months (22±5; Figure 6C: *P<0.05, 4–6 weeks versus 18 months; Student t test).

Discussion
In this study, we found that age influences the degree of hemodynamic compromise and collateral outgrowth in a model of unilateral cerebral hypoperfusion in C57/BL6 mice. The higher hemodynamic compromise until Day 7 in aged animals was in line with morphological findings and 14 days after unilateral ICA occlusion aged mice reached the same functional recovery as young animals despite less anatomic adaptation. Our results suggest that arteriogenic adaptations merely accelerate CVRC recovery and in this context, impaired vascular reactivity may be a target for novel therapeutic rescue strategies in the acute phase of hemodynamic stroke.

Functional Relevance of the P1 Segment Distribution After Proximal Vessel Occlusion
The patency of the P1 segment is a crucial factor for flow redistribution between the anterior and posterior circulation after occlusion of main conductance vessels and is described

Figure 4. Age influences the degree of hemodynamic compromise after unilateral ICA occlusion in C57/BL6 mice. A, Typical CBF response to 50 mg/kg acetazolamide after sham (left panel) and unilateral (right) ICA occlusion (ICAO; right panel). B, Laser speckle images of relative cortical perfusion before and after acetazolamide challenge in sham and ICAO animals; the limited perfusion increase in the region of interest (ROI=dashed rectangle) after acetazolamide in animals with ICAO can be noted in the right panels. C, Bar graph illustrating the total mean CVRC in individual groups. In all age groups, ICAO resulted in a significantly lower CVRC compared with sham-operated animals (*P<0.01). D, Line graphs comparing the mean CVRC in sham (upper graph) and ICAO (lower graph) animals over the course of the 14-day monitoring period. The similar functional compensation of mice aged 18 months compared with animals aged 4 to 6 or 12 weeks despite a markedly higher hemodynamic impairment in aged animals is depicted in the lower graph (*P<0.05 “4–6 weeks” sham versus ICAO; **P<0.01 “18 months” sham versus ICAO; §P<0.01 “ICAO” 18 months versus 12 weeks; #P<0.05 “ICAO” 18 months versus 4–6 weeks). ICA indicates internal carotid artery; CBF, cerebral blood flow; CVRC, cerebrovascular reserve capacity; R, right hemisphere.
ICA occlusion might be functionally negligible. In a model of mild hemodynamic impairment by unilateral occlusion, we provide evidence that individual P1 segment characterization might not be a reliable predictor for sufficient collateral flow between the anterior and posterior circulation after uni- or bilateral ICA occlusion, although all animals showed a prominent interconnection between both hemispheres by the anterior communicating artery complex. Therefore, our results confirmed the variable native P1 segment anatomy, ranging from absent to uni- and bilateral P1 segment development. However, we found that just “patency” of the P1 segment is not a reliable predictor for sufficient collateral flow between the anterior and posterior circulation after unilateral or bilateral ICA occlusion (Figure 1). In our study, 2 of 20 animals (10%) exhibited large-type P1 interconnections (diameter >100 μm) and bilateral ICA occlusion, these animals showed a perfusion drop to merely 56%±0.1% of baseline perfusion compared to 36%±8% in animals with a P1 segment diameter of <100 μm. This is in line with previous findings in which the P1 segment interconnection influenced the severity of acute forebrain ischemia after bilateral common carotid artery occlusion in the mouse.20,22,23 However, the perfusion after a mild reduction of flow in the anterior circulation by unilateral ICA occlusion was not influenced by the P1 segment heterogeneity. This was mainly due to the fact that all animals showed a prominent interconnection between both hemispheres by the anterior communicating artery complex. Therefore, our results provide evidence that individual P1 segment characterization in a model of mild hemodynamic impairment by unilateral ICA occlusion might be functionally negligible.

Characterization of Hemodynamic Compromise and Outward Collateral Remodeling After Unilateral ICA Occlusion in C57/BL6 Mice Aged 4 to 6 Weeks

Vasodilatation is a fundamental rescue mechanism to prevent harmful effects of proximal vessel occlusion. Nonischemic cerebral hyperperfusion is typically characterized by a normal or at best mild reduction of resting CBF and implies an impaired CVRC. Baseline CBF measurements alone may not sufficiently predict the risk of ischemia, which was demonstrated in patients with symptomatic carotid artery occlusion.25 In such cases, measurement of the CVRC is a powerful tool for risk assessment of ischemic stroke.5,6 Therefore, experimental studies aimed at investigating cerebral arteriogenesis should integrate functional CBF assessment because restoration of CVRC is a result of augmented collateral flow.26,27

In the rat, 3-vessel occlusion was reported to abolish ipsilateral CVRC with a functional recovery to approximately 50% within 3 weeks.18 Thirty minutes (Day 0) after permanent unilateral ICA occlusion in C57/BL6 mice aged 4 to 6 weeks, we found a significant hemodynamic impairment over the ipsilateral hemisphere (44% compared with age-matched sham animals), which lasted until Day 7 after the procedure (75%) and reached functional recovery by Day 14 (Figure 4C). In the rat, functional recovery coincided with collateral growth primarily at the P1 segment (the interconnection between the anterior and posterior circulation of the circle of...
Willis)\textsuperscript{13,15} and the leptomeningeal anastomoses.\textsuperscript{13} In C57/BL6 mice, unilateral ICA occlusion resulted in a striking external diameter increase in nearly all vessel segments of the circle of Willis. The most pronounced increase was noted at the hemispheric interconnection in the anterior circulation (ACA segment) and positive outward remodeling was confirmed by a marked proliferation of endothelial and perivascular cells. No diameter increase was detected in the P1
segment(s) of the PCA (Figure 5A), which is in line with our finding regarding the negligible P1 segment influence on cortical perfusion and suggests a different primary rerouting of blood flow in the C57/BL6 mouse compared with the rat.

In regard to the leptomeningeal vasculature after ICA occlusion, we noted an increase in larger caliber anastomoses (25–35 μm) at the cost of smaller caliber vessels (5–25 μm; Figure 6A). These diameters were presented in the form of frequency distributions to illustrate that the diameter increase was most likely due to positive remodeling of the pre-existing anastomoses, which may increase the total cross-sectional vessel area and, thus, the total conductance of the organ vasculature. Interestingly, ICA occlusion did not lead to a difference in the total number of leptomeningeal anastomoses. Here, the reports in the literature are inconsistent. In general, our observations are in line with the findings of Todo and colleagues,28 who performed unilateral carotid artery occlusion in C57/BL6 mice and also found an increase in the diameter but not the number of leptomeningeal anastomoses. However, a previous study from our group based on a rat model of chronic MCA, and PCA segments after unilateral or bilateral carotid surgery. In addition, the different perfusion pressure gradients and fluid shear stress between the leptomeningeal ACA, MCA, and PCA segments after unilateral or bilateral carotid artery occlusion showed an increase in the total number of anastomoses, whereas the diameter of those vessels remained unchanged.13

In contrast to the present study, a different species was investigated and animals were perfused 6 instead of 3 weeks after surgery. In addition, the different perfusion pressure gradients and fluid shear stress between the leptomeningeal ACA, MCA, and PCA segments after unilateral or bilateral carotid artery occlusion may be responsible for different mechanisms of leptomeningeal outgrowth.

Next, we looked for alterations of the cerebrovascular network beyond the basal and leptomeningeal vasculature and found a 21% increase in the number of intraparenchymal vessels displaying anti-SMA immunoreactivity (Figures 2B and 2D). Expression of anti-SMA has been demonstrated with early angiogenic process microvessel maturation, especially in arterioles,29 and regulation of the cerebrovascular reactivity mainly occurs at the level of precapillary arteriolar resistance vessels. Apart from basal and leptomeningeal collateral outgrowth, this finding may represent an additional morphological correlate to the restored CVRC that we noted.

We then analyzed the parenchymal capillary density and found a slight increase in capillary density that was accompanied by a mild decrease in pericyte coverage by the same percentage (Figures 2B and 2D). Given the fact that this is the first study to investigate parenchymal microvascular morphology after permanent ICA occlusion and the size of our histological series (n=5), these findings should be interpreted with caution. One might argue that the increased microvascular density could be an expression of posts ischemic angiogenesis.30 However, hypoxic tissue injury was excluded in our pilot experiments and has been reported with an incidence of merely 1.5% in cases in which baseline CBF after common carotid artery occlusion dropped below 35%.31 Also, we did not detect a difference in microvascular density in the hypoxia sensitive CA1 area of the hippocampus or the caudate putamen (data not shown). Therefore, our data rather point to an activation of parenchymal angiogenesis, because the reduction of the vascular desmin coverage could be the expression of transition from a mature to a more plastic microvasculature. A possible mechanism could be mediation by the angiopoietin system, which was shown to control stages of the angiogenic cascade related to assembly, maturation, and quiescence32 and in particular angiopoietin-2 may act as a vessel destabilizing agent that induces permeability and leads to dissociation of cell–cell contacts.33 Future experiments in the line of vessel permeability, blood–brain barrier evaluation, and quantitative CBF assessment could shed light on the structural integrity of these microvessels and their role in microcirculatory flow redistribution.

The Limitation of Baseline Perfusion Assessment
for the Purpose of Characterizing Cerebral Hemodynamic Impairment in C57/BL6 Mice

In contrast to Kitagawa and colleagues,31 who determined a sustained 60% drop of baseline CBF by Laser Doppler Flowmetry after permanent unilateral common carotid artery occlusion in the C57/BL6 mouse, we performed blood flow measurements by Laser Speckle Contrast Analysis after unilateral ICA occlusion. We intentionally developed a model with sustained flow through the external carotid artery to permit future investigations regarding augmentation of collateral flow after indirect revascularization techniques.34 Interestingly, ICA occlusion did not lead to a reduction of baseline perfusion compared with age-matched sham animals as early as 30 minutes after the procedure until the end of the monitoring period. To some extent, sustained collateral flow over the external carotid artery may have contributed to this effect. Furthermore, the different CBF measurement techniques could be responsible, because highly localized regional CBF changes may have been diluted by Laser Speckle Contrast Analysis. However, Laser Speckle Contrast Analysis depicts the hemodynamic situation in a cortical vascular territory more precisely because a more representative cortical perfusion map can be obtained. Overall, our findings caution against relying on baseline perfusion measurements alone as a measure for hemodynamic impairment after permanent proximal vessel occlusion in C57/BL6 mice.

Age Influences Both the Degree of Hemodynamic Compromise and Outward Collateral Remodeling After Unilateral ICA Occlusion in C57/BL6 Mice

In general, cortical CBF is maintained by an adequate perfusion pressure in the proximal vasculature. After proximal occlusion of an artery distal to the circle of Willis, perfusion of the brain tissue depends, in part, on the extent of pre-existing leptomeningeal anastomoses. Recently, the native leptomeningeal collateral circulation has been identified as a major determinant in the severity of experimental stroke.13,35 Rarefaction of leptomeningeal anastomoses coincides with an increased vascular resistance that may hamper flow redistribution between vascular territories. Similar leptomeningeal rarefaction with age has previously been reported16 and was confirmed by our results. The 27% decrease in the number of leptomeningeal anastomoses in sham mice aged 18 months was in line with a 20% reduction of baseline perfusion in these animals. Despite the fact that additional
ICA occlusion did not lead to a drop in baseline perfusion in these animals, the loss of leptomeningeal collaterals should not be considered as functionally negligible. Particularly in the setting of chronic hemodynamic impairment, leptomeningeal collateralization may represent the last line of defense against acute ischemic tissue injury. A limitation of this study remains that we cannot completely exclude that the higher baseline perfusion in mice aged 4 to 6 weeks compared with animals aged 18 months could be influenced by the naturally higher CBF during adolescence in the 4- to 6-week age group. However, we obtained a true “adult” reading in a group of mice aged 12 weeks, which was in line with our results in animals aged 4 to 6 weeks.

An unexpected finding was that despite similar basal and leptomeningeal vessel diameters in old and in young sham mice, animals aged 18 months were already more severely hemodynamically impaired than young mice as early as 30 minutes after ICA occlusion. Because aged animals nevertheless reached the same functional recovery as young animals by Day 14, this suggests (1) that a functionally impaired vascular response might be the primary mechanism of the more severely impaired CVRC in aged mice; and (2) that arteriogenic adaptations merely accelerate CVRC recovery.

In the elderly, changes that contribute to the risk of vascular dysfunction and predispose toward cardio- and/or cerebrovascular disease are vascular inflammation, endothelial dysfunction, increased arterial stiffness, endothelial apoptosis, cellular senescence, and oxidative stress. For example, age-related oxidative stress in the coronary circulation and other vascular beds hampers the bioavailability of nitric oxide with severe impairment of flow-/shear stress-induced vasodilation in response to tissue oxygen demand. The impairment of nitric oxide bioavailability, an age-related decline in endothelial nitric oxide synthase expression, and other factors may in conjunction promote functional impairment of vascular reactivity, vascular inflammation, and arteriosclerosis. Against this background, future experimental studies could test whether therapeutic application of vasodilators during the acute phase of hemodynamic stroke alleviates ischemic tissue injury in aged mice.

Faber and colleagues recently demonstrated an age-associated reduced recovery of perfusion and impaired collateral remodeling in the hindlimb ischemia model. In the brain, our study provides first evidence of an “age-dependent” effect on collateral remodeling during chronic hypoperfusion. In contrast to young animals in our study, unilateral ICA occlusion in aged mice resulted in a significant diameter increase merely in the ACA segment of the anterior circulation and did not lead to a significant diameter change of the leptomeningeal anastomoses. Together with the fact that unilateral ICA occlusion did not influence baseline CBF despite leptomeningeal rarefaction in aged mice, our findings underline the principal role of the anterior communicating artery complex for flow redistribution after proximal ICA occlusion in the C57/BL6 mouse. Collateral remodeling is initiated by increased fluid shear stress after arterial obstruction followed by recruitment of hematopoietic cells to the pericollateral region, where they release cytokines and growth factors that direct remodeling. Intriguingly, evidence in the hindlimb ischemia model suggests that age-associated impaired remodeling is not due to reduced leukocyte recruitment. Although investigation of the mechanisms involved were beyond the scope and purpose of our study, a possible explanation for the hampered collateral remodeling with age could be that the shear-stress mediated increase in endothelial nitric oxide synthase expression is reduced in aging as are hypoxia-inducible factor-1 and vascular endothelial growth factor, each normally contributing to collateral remodeling and serving as a collateral maintenance factor.

Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Cerebral hemodynamic reserve and vascular remodeling in C57/BL6 mice are influenced by age

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Supplemental Methods

Measurement of cerebral perfusion changes after proximal vessel occlusion in dependence of the P1 segment distribution

In n=20 C57/BL6 mice aged 4-6 weeks, two Laser Doppler Flowmetry (LDF) probes (1mm diameter, Moor Instruments, Devon, England) were bilaterally mounted over the middle cerebral artery (MCA) territory (3mm dorsal to the bregma and 3mm lateral to the midline) and connected to an LDF monitor (DRT4, Moor Instruments, Devon, England). The probes were secured with dental resin (Paladur, Heraeus Kulzer, Hanau, Germany) and the animal was turned to supine position. The internal carotid artery (ICA) was bilaterally exposed through a midline neck incision. Temporary uni- and bilateral ICA-occlusion was performed for 20 seconds in an alternating fashion with microsurgical clips (Aesculap, Tuttlingen, Germany) and repeated 3 times during which the percent decrease of cerebral perfusion was recorded. After each occlusion procedure a recovery period of 4 minutes was allowed. Next, each animal was perfused with a latex/carbon black compound (see below) in order to visualize the P1 segment connection between the anterior and posterior circulation. Presence or absence of individual P1 segments was determined under a surgical microscope at x40 magnification. The mice were grouped according to their P1 segment distribution (absent, ipsilateral, contralateral or bilateral P1 segment, in regard to the site of LDF registration) and the percent decrease of cerebral blood flow (CBF) after ICA-occlusion was calculated in each group.

Measurement of relative cerebral perfusion by Laser Speckle Contrast Analysis

Experimental CBF measurements by LDF are hampered by highly variable contact properties. The difficulty of probe placement at precisely the same area and the low spatial resolution limit the comparability and interpretation of experimental results. Laser Speckle Contrast Analysis (LASCA) was proven to be a valid technique for transcranial imaging of relative cortical CBF with high spatiotemporal resolution under normal and ischemic conditions in mice. The laser penetration depth of LASCA depends on the optical properties of the laser light and the sampled tissue and is reported in the range of 500μm – 1000μm.
In order to ensure stable baseline CBF-Flux measurements and avoid hemodynamic fluctuations, all LASCA measurements in our study were performed 30 minutes after induction of anesthesia. To ensure optimal optical properties, the scull was coated with a thin layer of sterile paraffin oil. The LASCA device was positioned 20cm above and perpendicular to the cortical surface and direct illumination of the surgical field by light sources other than the laser light was avoided.

*Tissue preparation for repeated transcranial Laser Speckle Contrast Analysis*

The tissue preparation gets more demanding after repeated opening and suturing of the scalp for consecutive measurements in the same animal. The formation of scar and inflammatory tissue may influence the LASCA measurement and was minimized by the following measures:

1. The scalp was shaved and disinfected with rubbing alcohol before surgery.
2. All surgical instruments were sterilized and sterile gloves were worn during surgery.
3. The scalp was mobilized by gentle retraction and scissor/microscissor dissection superficial to the fascia of the temporal muscle in order to minimize bleeding.
4. Fibrous adhesions and scar tissue on the scull surface after repeated opening of the scalp were carefully dissected and removed. Haemostasis was accomplished by sterile saline irrigation and cotton swab compression.
5. Suturing was performed with 6/0 sterile nylon and the scalp was disinfected after surgery.

*Latex/Carbon Black Perfusion*

Under deep anesthesia, an aortal catheter (internal diameter 0.76mm, Vasofix Braunuele, Braun, Melsungen, Germany) was inserted and a sub-lethal dose of 50mg/kg papaverine hydrochloride was injected. After 60 seconds, the vena cava was incised to allow venous outflow and 1ml sterile saline was injected. Immediately after the saline injection, a white liquid latex compound (Chicago Latex Product no. 563, Chicago Latex Products, Crystal Lake, USA) mixed with 20µl/ml carbon black (Derussol N25/L, Degussa, Frankfurt, Germany) was infused into the aorta at 150 mmHg for 5
minutes. The animals were placed in ice-cold water for 20 minutes to ensure hardening of the latex. For assessment of collateral artery growth on day 21 after surgery, the brains were carefully removed and photos of the leptomeningeal and basal vasculature were taken at x20 magnification. Leptomeningeal anastomoses were defined at their point of confluence between the distal MCA and the distal anterior cerebral artery (ACA) or between the distal MCA and the posterior cerebral artery (PCA).

**Immunohistochemistry**

The cerebral vasculature was detected by a rat anti-mouse CD31 antibody (1:50 dilution in 0.5% Casein; PECAM-1, BD Biosciences, Franklin Lakes, NJ, USA) and a donkey anti-rat IgG conjugated with CyTM3 (1:200 dilution in 0.5% Casein; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibody. Pericytes were detected with two different antibodies: a rabbit anti-desmin polyclonal antibody (Dilution 1:100 in 0.5% Casein; Abcam, Cambridge, UK) detected by CyTM5-conjugated donkey anti-rabbit IgG antibody (Dilution 1:100 in 0.5% Casein; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) and a monoclonal mouse anti smooth-muscle actin (SMA) directly labeled with FITC antibody (Dilution 1:250 in 0.5% Casein; Clone 1A4, Sigma-Aldrich, St. Louis, Missouri, USA). For quantification of vessel density and pericyte coverage, three random and un-overlapping fields (x200 magnification) were selected in the cortical MCA territory of each hemisphere in each cryosection (four sections per animal, five animals per group). Blood vessels were defined by positive CD31 staining. Any CD31 positive cell or CD31 positive cell cluster that clearly separated from adjacent microvessels was considered as a single and countable microvessel.

For cell proliferation analysis, the cerebral vasculature (CD31) and pericytes (anti-SMA) were detected by the method mentioned above. Cell proliferation was visualized with a monoclonal rabbit anti-mouse Ki67 antibody (Dilution 1:200 in 0.5% Casein; Thermo Scientific, Waltham, MA, USA) detected by CyTM5-conjugated donkey anti-rabbit IgG (Dilution 1:100 in 0.5% Casein; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). In each cryosection (four sections per animal, three animals per group at each time point), the corresponding basal vessel segment (ACA,
MCA, ICA, posterior communicating artery (PcomA), PCA) and the leptomeningeal vessels were inspected regarding perivascular and endothelial cell proliferation.

Following the results of the cell proliferation analysis, an H&E stain was performed on day 7 for analysis of the basal vessel wall diameter of the ACA and MCA at bregma level ±0.0mm after sham or ICA-occlusion. In each section (one section per animal, three animals per group) the ACA and MCA were identified at the base of the brain and the vessel wall diameter was measured at 4 opposing points using freeware image analysis software (ImageJ64, http://reweb.nih.gov/ij/). From this data, a mean vessel wall diameter was calculated for the ACA and MCA in Sham and ICA-occlusion groups.

Statistical analysis

All Data are presented as mean±SD. To compare the percent decrease of CBF in dependence of the P1 segment distribution, a one-way ANOVA procedure was performed. For LASCA comparison of baseline perfusion and cerebrovascular reserve capacity (CVRC) after right ICA-occlusion and sham procedures, a two-way ANOVA for repeated measures with subsequent pair wise comparison of means by Fisher’s least projected difference test was performed. Basal vessel diameters were compared with a one-way ANOVA procedure. The relative frequency of leptomeningeal vessel diameters was compared by a two-way ANOVA procedure with subsequent Bonferroni analysis. Differences in the total number of leptomeningeal anastomoses were tested by a student’s t-test. The CD31/anti-SMA vessel diameter frequency distribution was analyzed with a two-way ANOVA procedure with subsequent Bonferroni analysis. For histological analysis of basal vessel wall diameter, CD31 vessel density, Desmin coverage and anti-SMA vessel density, student’s t-tests were performed. All statistics were generated with GraphPad Prism for Mac (Version 5.0d, GraphPad Software, San Diego California, USA). Statistical significance was set at p<0.05.
Supplemental Figure S1:

Positive remodeling of the basal vasculature in the anterior circulation after unilateral ICA-occlusion (ICAO) in C57/BL6 mice aged 4-6 weeks.

(A, B) H&E stain at the level of the middle cerebral artery (MCA, bregma level ±0.0) on day 7 after sham (A) or unilateral ICAO (B). (C) The vessel wall diameters of the ACA and MCA segments were determined at 4 opposing points of the vascular wall in 3 animals per group. On day 7 after ICAO (time point where the most pronounced proliferation activity was determined by Ki67; for reference, please see Figure 2A) we determined a significant mean vessel wall diameter increase to 14.8±3µm in the ACA (Sham: 11.2±1µm) and 10.4±1µm in the MCA (Sham: 7.1±2µm) as further confirmation of positive outward remodeling (Bar = 100µm; p<0.05).

Supplemental references
