Neuroimaging Biomarkers Predict Brain Structural Connectivity Change in a Mouse Model of Vascular Cognitive Impairment

Philipp Boehm-Sturm, PhD*; Martina Füchtemeier, PhD*; Marco Foddis; Susanne Mueller; Rebecca C. Trueman, PhD; Marietta Zille, PhD; Jan Leo Rinnenthal, PhD; Theodore Kypraios, PhD; Laurence Shaw, PhD; Ulrich Dinnagl, MD; Tracy D. Farr, PhD

Background and Purpose—Chronic hypoperfusion in the mouse brain has been suggested to mimic aspects of vascular cognitive impairment, such as white matter damage. Although this model has attracted attention, our group has struggled to generate a reliable cognitive and pathological phenotype. This study aimed to identify neuroimaging biomarkers of brain pathology in aged, more severely hypoperfused mice.

Methods—We used magnetic resonance imaging to characterize brain degeneration in mice hypoperfused by refining the surgical procedure to use the smallest reported diameter microcoils (160 μm).

Results—Acute cerebral blood flow decreases were observed in the hypoperfused group that recovered over 1 month and coincided with arterial remodeling. Increasing hypoperfusion resulted in a reduction in spatial learning abilities in the water maze that has not been previously reported. We were unable to observe severe white matter damage with histology, but a novel approach to analyze diffusion tensor imaging data, graph theory, revealed substantial reorganization of the hypoperfused brain network. A logistic regression model from the data revealed that 3 network parameters were particularly efficient at predicting group membership (global and local efficiency and degrees), and clustering coefficient was correlated with performance in the water maze.

Conclusions—Overall, these findings suggest that, despite the autoregulatory abilities of the mouse brain to compensate for a sudden decrease in blood flow, there is evidence of change in the brain networks that can be used as neuroimaging biomarkers to predict outcome. (Stroke. 2017;48:468-475. DOI: 10.1161/STROKEAHA.116.014394.)

Key Words: biomarkers ■ diffusion tensor imaging ■ hypoperfusion ■ magnetic resonance imaging ■ mouse ■ neuroimaging ■ vascular cognitive impairment

Vascular risk factors are thought to be the pathological initiation point in the development of vascular cognitive impairment.1 There are many vascular cognitive impairment subtypes with a variety of radiological features ranging from microbleeds and lacunar infarctions to diffuse white matter hyperintensities. Progression of white matter hyperintensities is highly predictive of cognitive decline.2 The mouse model of bilateral carotid artery stenosis attracted attention when the authors reported rarefaction and degeneration of the corpus callosum after 30 days of brain hypoperfusion.3 A follow-up study reported that hypoperfused mice exhibited cognitive deficits, specifically spatial working memory impairments in the radial arm maze.4 Similar deficits were reported using the same model, but with a more subtle pathology that instead includes small accumulations of degraded myelin basic protein and a change in the distribution of myelin-associated glycoprotein in several white matter structures.5 Another group has observed a loss of fluoromyelin staining and an increase in matrix metalloproteinase-9 in the corpus callosum of hypoperfused mice, which can be prevented with free radical scavengers.6,7 Whether or not this range of pathological changes are detectable with neuroimaging is less clear. Decreases in fractional anisotropy (FA), using diffusion tensor imaging (DTI), were observed in white matter,8 which became more...
pronounced with 6 months of hypoperfusion. Our group has failed to observe FA decreases in hypoperfused mice, though we have reported alterations in several other quantitative DTI parameters. In our hands, this model has been both behaviorally and histologically mild, which might reflect inherent variability of the model, or the fact that rodents exhibit a high degree of recovery and compensation. Therefore, the purpose of this study was 2-fold. First, we aimed to increase the severity of hypoperfusion using a refined surgical approach in aged mice, to improve the phenotype. Second, we aimed to identify neuroimaging biomarkers of degeneration with focus on graph theory to our DTI data to predict cognitive decline.

Methods
Experiments were approved by the Landesamt für Gesundheit und Soziales and conducted according to the German Animal Welfare Act and institutional guidelines. Twenty-four male C57/BL6 J mice (purchased at 8 weeks of age, Charles River, Germany) were housed in a temperature (22±2°C), humidity (55±10%), and light (12/12-hour light/dark cycle) controlled environment. Unexpectedly, 1 animal needed to be humanely euthanized before randomization in accordance with our animal permissions because of ill health and substantial weight loss. The remaining animals were later randomized between 9 and 13 months of age to undergo hypoperfusion (n=12) or sham (n=11). Hypoperfusion was induced by winding a custom ordered, nonmagnetic, surgical grade microcoil (160 μm inner diameter; Shannon Coiled Springs Microcoil, Limerick, Ireland) around one of the carotid arteries. The sham procedure was performed with a larger diameter microcoil (500 μm) that did not constrict the vessel. The muscles and glands were guided back into place, and local anesthetic was applied to the sutured wound before recovery. Twenty-four hours later, the same procedure was repeated on the other carotid artery. This delay represents an important refinement that does not result in higher mortality when using the smaller sized microcoils. All experimenters were blind, and all analysis was performed blind. Mice were imaged before and at 24 hours and 1- and 4-weeks post-surgery for estimation of cerebral blood flow (CBF) using arterial spin labeling. DTI, magnetic resonance spectroscopy, and angiography were acquired between 5- and 7-weeks post-surgery. The novel object recognition test was conducted 1 week before and between 4- and 5-weeks post-surgery. At 6 weeks, animals were trained in the Morris water maze, and tissue was processed for immunohistochemistry. Detailed methods are available in the online-only Data Supplement.

Results
Deficits in CBF After Hypoperfusion Recover and Are Accompanied by Changes in the Cerebrovasculature and Hippocampal Degeneration
CBF remained stable across all time points in the sham group but decreased by more than half within 24 hours of surgery in the hypoperfused group. Within 1 week, CBF began to increase in the hypoperfused group; it continued to increase but was still significantly different from shams at 4 weeks (Figure 1A and 1B, main effect of time F(2.3,43.3)10.212; P=0.0001; and group F(1,19)36.977; P=0.0001; and interaction F(2.3,43.3)10.240; P=0.0001). This suggests that the hypoperfused mouse brain is autoregulating in response to a decrease in CBF. Angiography at 7 weeks (Figure 1A) revealed increased tortuosity in the Circle of Willis, which may be partially responsible for the recovery in CBF. However, the overall size of the Circle of Willis vasculature was not significantly different between groups (Figure 1A in the online-only Data Supplement). There was no

Figure 1. Cerebral blood flow (CBF) and morphological changes in the hypoperfused brain. A, Representative CBF maps and angiography reconstructions showing increases in Circle of Willis tortuosity in hypoperfused mice. B, CBF over time (means±SD) in sham (n=10) and hypoperfused (n=11) groups. C, % hippocampal size in both groups.
significant difference in ventricle:brain ratio (Figure 1B in the online-only Data Supplement), but there was a decrease in hippocampal size in the hypoperfused mice, though this failed to achieve significance (Figure 1C; Student unpaired t test \( t(19)=2.068; P=0.053 \)).

Metabolite concentrations were measured in the striatum with magnetic resonance spectroscopy (Figure 2), but there were no significant concentration differences in any of the metabolites (Figure 2B).

**Hypoperfused Brain Exhibits Little Evidence of Pathology With Diffusion Index Measures but Undergoes Substantial Network Reorganization**

Seven of the 23 brain regions from the DTI data were selected for analysis: anterior commissure, corpus callosum, and internal capsule, as well as gray matter that may be affected by hypoperfusion or is presumed to be involved in cognitive decline: hippocampus, striatum, cingulate cortex, and thalamus. FA, mean diffusivity, axial diffusivity, and radial diffusivity values are depicted for both groups in 2 white and 1 gray matter structure (Figure 3A through 3D). FA and axial diffusivity decreases are correlated with tissue degeneration and axonal degradation, whereas mean diffusivity and radial diffusivity increases are correlated with loss of tissue integrity and demyelination.\(^{11}\) There were no significant differences between groups for global efficiency (Figure 4A), suggesting that the overall ability of the brain network to exchange information was minimally affected by hypoperfusion. However, significant group differences were noted in several measures of functional segregation. Modularity was significantly lower in the hypoperfused group (Student unpaired t test \( t(17)=3.028; P=0.014 \); Figure 4B), which suggests a reduced number of heavily interconnected subgroups of structures. Interestingly, several lower level clustering parameters were significantly higher in the hypoperfused group: transitivity (Student unpaired t test \( t(17)=-4.706; P=0.0007 \); Figure 4C), clustering coefficient (Mann–Whitney U test: \( U=9, z=-2.939; P=0.007 \); Figure IIA in the online-only Data Supplement), and local efficiency (Student unpaired t test: \( t(17)=-3.410; P=0.007 \); Figure 4D). These 3 parameters were also highly correlated. Transitivity and clustering coefficient rely on the presence of triangles (groups of 3 connected brain structures). This reflects an increase in the total number of connected triplets, as well as the prevalence of clustering in general and an increased ability of small subnetworks to function when any given node is removed. No significant differences were noted for degrees or assortativity (Figure IIB and IIC in the online-only Data Supplement).

A logistic regression model from the network parameters was fitted to the data. The response variable was group classification, and the covariates were the different network parameters. We performed model selection by exploring all possible models and chose the 1 with the minimum Akaike Information Criterion, which penalizes models with too many parameters.
The best model showed that global and local efficiency and degrees could accurately classify the 2 groups of mice. Cross validation (by removing one animal at a time) showed that 15 of 19 animals were still correctly classified.

Reactive Gliosis Was Observed in the Hippocampus of Some Hypoperfused Mice Without Overt Rarefaction of the White Matter

We observed no evidence of white matter rarefaction with Luxol blue stain (Figure 5). There was also no evidence of increased astrocytic or microglial activity in any of the white matter structures. However, there was some reactive gliosis and scarring in the hippocampus of 2 of the hypoperfused mice.

Hypoperfusion Reduces Spatial Learning in the Water Maze, and Performance Is Correlated With Network Analysis Measures

Escape latencies decreased over the testing period (Figure 6A, main effect of time F(3.9, 80) = 13.2; P = 0.0001), and mice travelled decreasing distances to the platform (Figure IIIA in the online-only Data Supplement; main effect of time F(6, 108) = 16.1; P = 0.0001), indicating that both groups acquired the paradigm. However, hypoperfused mice exhibited slightly longer escape latencies than shams (Figure 6A, main effect of group F(1, 18) = 6.6; P = 0.01) and travelled greater distances to find the platform (main effect of group F(1, 18) = 4.7; P = 0.04), indicating a reduced rate of learning. There were no significant differences in swim speeds between groups (Figure 6B, no main effect of time F(3.17, 57.183) = 0.709; P = 0.558 or group F(1, 18) = 1.049; P = 0.319), suggesting that motor function was intact. There were no significant differences in the time spent in the target quadrant during the probe trial (Figure IIIB in the online-only Data Supplement). Furthermore, overall performance across the 7 days in the water maze was correlated with several network parameters: degrees, global and local efficiency, and clustering coefficient (Pearson correlation coefficients of 0.282, 0.285, and 0.364; P ≤ 0.069).

During the first trial in the novel object recognition test, discrimination ratios were ≈ 0.5, indicating that both groups explored the 2 objects equally. This occurred both before surgery (0.49 ± 0.03 [sham] and 0.53 ± 0.07 [hypoperfused]) and at 4-weeks post-surgery (0.55 ± 0.1 [sham] and 0.59 ± 0.1 [hypoperfused]). Discrimination ratios increased during the second trial (values > 0.5 = greater novel object exploration), indicating biased exploration of the novel object (Figure 6C). The
mean discrimination ratio of shams increased at 4 weeks and decreased in the hypoperfused group when compared with baseline. However, there was no significant effect of time or group (Figure 6C; F(1,17)0.0001; P=0.983 and F(1,17)0.5; P=0.48, respectively). This might reflect excessive exposure over time to the testing environment. For example, discrimination ratios on the second trial varied widely at 4 weeks (Figure 6C) because some animals explored very little (1 sham animal performed no exploration [data entered as missing]). Indeed, overall % time exploring the objects decreased in both groups at 4 weeks (5.6±1.4 [sham] and 4.7±1.2 [hypoperfused]) when compared with baseline (7.5±1.2 [sham] and 8.2±2.6 [hypoperfused]). When combined, exploration time was significantly lower at 4 weeks (Student paired t test t(19)4.0; P=0.001).

**Discussion**

This study demonstrates that CBF increases with time in this mouse model of hypoperfusion, which may be because of increases in tortuosity of the Circle of Willis. Although no brain atrophy was observed (ventricle:brain ratio), there was hippocampal shrinkage in the hypoperfused mice and reactive astrogliosis in the hippocampus of 2 mice. This was combined with spatial learning impairments in the water maze. We did not observe white matter rarefaction in the hypoperfused animals with basic histology, though this is
not surprising, because the pathology has been reported to be more subtle. The hypoperfused brain had clearly reorganized because application of graph theory to structural connectivity data showed an overall reduction in the ability to subdivide the brain into distinctive groups of highly interconnected structures, combined with an increase in connectivity among clusters of brain structures. Some network parameters were correlated with water maze deficits, and others were excellent predictors of outcome.

We reported a decrease in CBF that increases with time that was comparable to our previous results, as well as findings from the article that introduced this model: CBF recovered to within 10% of presurgical levels by 30 days. Similar results have also been reported with CBF recovery to 81.7% at 1 month and to 80% as early as 14 days after hypoperfusion. All of the previous studies used 180-μm-diameter microcoils made from piano wire. We have found that this material dissolves after implantation (Figure IV in the online-only Data Supplement) and could have explained the CBF recovery reported in these studies. However, the present study used a smaller diameter microcoil (160 μm) made from nonmagnetic, surgical grade material that was recovered at the conclusion of the experiments (Figure IV in the online-only Data Supplement), and near recovery of CBF was still observed in the hypoperfused group that occurred to a lesser extent than when 180 μm coils were used (Figure V in the online-only Data Supplement). We observed increases in the tortuosity of the Circle of Willis with angiography that may be partially responsible for the recovery in CBF because this collateral structure attempts to compensate. We did not observe an increase in Circle of Willis size; therefore, we hesitate to conclude that tortuosity equals arteriogenesis, though it is possible this phenomenon is playing a role in the observed CBF increase. We also substantially reduced interindividual variation and confirmed no changes in sham CBF, by using nonmagnetic microcoils in this study. At least when using magnetic resonance imaging, this strategy is necessary because the magnetic microcoils made from piano wire compromise CBF measurements by interfering with the magnetic resonance imaging acquisition.

Spatial working memory impairments have been reported in hypoperfused mice in the radial arm maze; animals displayed an increase in the number of revisiting errors after 1, 4, and 8 months of hypoperfusion. Spatial learning deficits in the water maze had not been previously observed until hypoperfusion was extended to 6 months. Compared with this, we increased the severity of hypoperfusion and thus may have achieved deficits sooner. Consistent with the prevailing hypothesis, it is possible that these memory deficits are because of white matter damage. However, it is also possible that the deficits are because of hippocampal damage. We observed a nonsignificant decrease in hippocampal size in this model, along with reactive astrogliosis in 2 hypoperfused mice. This is in line with 1 report that observed this in the most severely deprived hemisphere (with 160 μm inner diameter microcoils) when the degree of hypoperfusion was varied. Other groups have reported hippocampal damage after hypoperfusion. Working memory in the radial arm and water maze has a strong spatial component that is particularly sensitive to hippocampal damage. We chose the novel object recognition task to avoid this as much as possible; this task requires intact perirhinal and prefrontal cortices. There was decreased novel object exploration in the hypoperfused mice after 4 weeks when compared with baseline, but this was not significant. We did observe high variability in overall exploration at 4 weeks (compared with baseline) that we think is because of mice being overly habituated to the test environment; indeed, they spent less time exploring the objects in
There are a few recent reports that examine connectomes of the intact rodent brain.22,23 Our strategy to use aged mice and increase the severity of hypoperfusion resulted in a more reproducible phenotype when the smaller (160 μm) microcoils were implanted into naïve mice. Graph theory is a popular mathematical technique that models the complex organization of the brain. It is a novel approach for studying structural and functional human brain connectivity with neuroimaging techniques and is gaining popularity as a way to understand the diseased brain.20,21 One potential limitation of the present study is that the mice were trained in 9 hole boxes; though they failed to reach criteria, it is possible that this acted as a form of enrichment and protected them against the more severe hypoperfusion procedure. Indeed, we have observed a more pronounced phenotype when the smaller (160 μm) microcoils were implanted into naïve mice.

Global efficiency is a well-defined measure reflecting the potential for functional integration of information.24 It is strongly related to the density of connections, and decreases are accepted as a marker of network deterioration. A decline in global efficiency has predicted the development of dementia in patients with small vessel disease.25 Although we failed to observe a group difference in this study, global efficiency was extremely reproducible and, along with local efficiency and degrees, was successfully able to classify our groups using the logistic regression model; therefore, it is a valuable biomarker for hypoperfusion. Measures of functional segregation refer to the existence of subnetworks composed of structures that are heavily interconnected and thus presumed to participate in specialized functions. Modularity is strongly related to the adaptability of a system and was significantly decreased in the hypoperfused group. This indicates fewer distinctive subnetworks. Local efficiency estimates how well a local subnetwork is connected to any particular structure. Interestingly, we observed an increase in local efficiency in the hypoperfused group, alongside an increase in clustering coefficient and transitivity (other measures of connectivity within smaller subnetworks of 3 regions). Similar increases have been reported in patients with small vessel disease, which were negatively correlated with cognitive performance, and interpreted as small changes in tissue microstructure within particular hubs.26 It is possible the results of this study may reflect some degree of compensation on the part of the hypoperfused brain: closely connected structures increase their connections in response to an overall decline in the connections between larger functional subnetworks. One limitation of this type of analysis is that it is highly dependent on the number and density of connections. However, there was no difference in the number of connections between groups. The lack of atrophy in the hypoperfused mice would also suggest that our results are biologically meaningful, despite not being fully explained. It is also possible that with a larger sample size, or a greater amount of data as this technique becomes more prevalent, we could validate the model. The hypertensive rat has been suggested as another animal model of vascular cognitive impairment because it exhibits recognition memory deficits and reduced white matter integrity without overt white matter lesions.27

Our strategy to use aged mice and increase the severity of hypoperfusion resulted in a more reproducible phenotype because subtle behavioral impairments were observed. We also successfully identified several novel neuroimaging biomarkers for use with this mouse model. Accurate CBF imaging can be used to stratify groups of mice, and DTI in particular provided several quantitative indices alongside useful network parameters that were accurately able to predict outcome.

**Sources of Funding**

This work was supported by the German Research Foundation (DFG; Exc 257, NeuroCURE Cluster of Excellence), the Federal Ministry of Education and Research (BMBF; 01EO801, Center for Stroke Research Berlin), the Herman and Lilly Schilling Foundation, and the European Commission (01EW1201, ERA-NET NEURON).
Disclosures
None.

References
Neuroimaging Biomarkers Predict Brain Structural Connectivity Change in a Mouse Model of Vascular Cognitive Impairment

Philipp Boehm-Sturm, Martina Füchtemeier, Marco Foddis, Susanne Mueller, Rebecca C. Trueman, Marietta Zille, Jan Leo Rinnenthal, Theodore Kypraios, Laurence Shaw, Ulrich Dirnagl and Tracy D. Farr

*Stroke*. 2017;48:468-475; originally published online January 9, 2017;
doi: 10.1161/STROKEAHA.116.014394

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/48/2/468
Free via Open Access

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2017/01/19/STROKEAHA.116.014394.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Supplemental methods

Animals and experimental design
All experiments were approved by the Landesamt für Gesundheit und Soziales and conducted in accordance with the German Animal Welfare Act. Twenty-four male C57/BL6 J mice (8 weeks of age, Charles River, Germany) were housed in a temperature (22 ± 2 °C), humidity (55 ± 10 %), and light (12/12 hr light/dark cycle) controlled environment. Animals were food and water restricted for approximately 5 months whilst being trained in 9-hole boxes. Unexpectedly, one animal needed to be humanely euthanized prior to randomization in accordance with our animal permissions due to ill-health and substantial weight-loss. Bias was reduced using a pre-generated sequence to randomize the order of the surgical procedures, and with a coin to assign mice between 9-13 months of age to undergo hypoperfusion (n=12), or the corresponding sham procedure (n=11). Sample size calculations were not performed as there was no available information regarding aged mice for the hypoperfusion procedure, however, group sizes were in line with previous literature in young animals. All experimenters were blind to the condition of the animals during testing and scanning and all analysis was performed in a blinded fashion. Mice were imaged repetitively (prior to and at 24 hrs, and 1 and 4 weeks post surgery) for quantitative estimation of cerebral blood flow (CBF). DTI, magnetic resonance spectroscopy (MRS), and angiography were acquired between 5-7 weeks post surgery. The novel object recognition (NOR) test was conducted one week prior to, and between 4-5 weeks post surgery. At 6 weeks, the animals were trained in a standard hidden platform (place) task in the Morris water maze and tissue was subsequently processed for immunohistochemistry.

Hypoperfusion via bilateral common carotid artery stenosis
Anaesthesia was achieved using isoflurane in a 70:30 nitrous oxide:oxygen mixture and core body temperature was maintained at 37 ± 0.2 °C with an automated rectal probe and heat blanket. A midline incision was made in the neck, and a carotid artery was carefully exposed. Hypoperfusion was induced by winding a custom ordered, non-magnetic, surgical grade microcoil (160 µm inner diameter, Shannon Coiled Springs Microcoil, Limerick, Ireland) around one of the carotid arteries. The sham procedure was performed with a larger diameter microcoil (500 µm) that did not constrict the vessel. The muscle and glands were guided back into place and local anaesthetic was applied to the sutured wound prior to recovery. Twenty- four hours later, the same procedure was repeated on the other carotid artery. This delay represents an important refinement that does not result in higher mortality when using the smaller sized microcoils. Regular diet was placed on the floor of the cage to assist with feeding, and animals were provided with 6 mg/mL of Paracetamol in the drinking water to assist with post-operative pain (one day prior to, and up to three days post-surgery). A tumor was discovered in 1 sham animal (subsequently excluded) (sham (n=10)). One hypoperfused animal was euthanized immediately after surgery (hypoperfused (n=11)). One sham animal died during DTI acquisition, and did not undergo spectroscopy, angiography, or behavioural testing (sham (n=9)).

MRI measurements
Anaesthesia was again achieved using isoflurane as per above, and body temperature and respiration rate were monitored with MRI compatible equipment (Small Animal Instruments, Inc., Stony Brook, NY).

Cerebral blood flow and angiography
CBF and angiography were measured on a 7 T Pharmascan using Paravision 5.1 software (Bruker BioSpin, Ettlingen, Germany). For the CBF measurement, radio frequency transmission was achieved with a 72 mm diameter quadrature resonator actively decoupled to a mouse quadrature surface coil used for reception (Bruker BioSpin, Ettlingen, Germany). A single slice (1 mm) flow-sensitive alternating inversion recovery (FAIR) sequence with a rapid acquisition with relaxation enhancement (RARE) readout was used (repetition time (TR)/recovery time/echo spacing (ΔTE)/effective echo time (TEeff): 12 000/10 000/7.2/35.9 ms, respectively, 16 inversion times (35-1500 ms), RARE factor: 32, inversion slice thickness: 4 mm, 180° hyperbolic secant (sech80) inversion pulse (20 ms), field of view (FOV): 25.6 mm², matrix: 128 x 64 enlarged by partial fourier transform to 128 x 128, resolution: 200 µm², 12 min).

For angiography measurements, a 20 mm diameter quadrature volume coil (RAPID Biomedical, Rimpar, Germany) was used for radio frequency transmission and reception and a 3D time of light (TOF) sequence was used (TR/TE: 15/2.5 ms, α: 20°, FOV: 25 mm², resolution: 98 x 130 x 196 µm³ zero-filled to 98 µm³, 6 min).

Spectroscopy, T2 weighted and diffusion tensor imaging
T2 weighted imaging, DTI, and MR spectra were acquired on a 7 T Biospec with a cryogenically cooled transmit/receive surface coil and Paravision 6.0 software (Bruker BioSpin, Ettlingen, Germany).

A 2D RARE T2 sequence was used for anatomical images (TR/ΔTE/TEeff: 3100/11/33 ms, RARE factor: 8, 29 consecutive slices, slice thickness 0.45 mm, FOV: (16.2 mm²), resolution: 100 µm², NA: 2, 2 min 4 s). An isotropic echo planar imaging (EPI) DTI sequence was used (TR/TE: 7500/18 ms, 4 segments, 2 b values (0 and 1000 s/mm²) with 5 b images, 126 gradient directions (1 experiment/direction), gradient duration (Δ) 2.5 ms, gradient separation (Δ) 8.1 ms, 58 consecutive slices, FOV: 16.2 mm², acquisition matrix 72 x 48 enlarged by partial fourier transformation to 72 x 72, resolution: (225 µm³), 1 h 5 min). Two animals, one from the sham and one from the hypoperfused group exhibited fluctuating respiration rates after the DTI
acquisition, did therefore did not undergo MRS (sham (n=8) and hypoperfused (n=10)) as. A stimulated echo acquisition mode (STEAM) sequence was used for spectroscopy following local shimming (MAPSHIM) across a cubic 8 mm³ voxel placed in the striatum (TR/TE/mixing time: 2500 ms/3 ms/10 ms, number of averages (NA): 256, VAPOR water suppression, 10 min 40 s).

Data Analysis

Cerebral blood flow and angiography

CBF maps were calculated using the Perfusion ASL macro in Paravision 5.1 software via the T₁ method using a blood T₁ value of 2100 ms and a brain blood partition coefficient of 0.89 mL/g. A custom written Matlab Release 2013a (MathWorks, Natick, MA, USA) script extracted the CBF maps from Paravision, and prompted manual delineation of regions of interest (ROI) in the striatum. The resulting CBF values were expressed in mL/min/100g.

Angiography images were analyzed as previously described. Briefly, the spatial dimensions or the raw data was up-scaled by a factor of ten, exported into FSL software (Analysis Group, FMRIB, Oxford, UK), and the FLIRT tool was used for co-registration. Registered images were exported into ImageJ freeware (National Institutes of Health) and a maximum intensity projection (MIP) of the Circle of Willis was prepared with a custom plugin. A threshold (14 000 in 16 bit images, i.e. ~43% of max) was used to create a binary image of the MIP so that the number of voxels in the Circle of Willis could be counted and expressed in μm².

Spectroscopy and T₂ weighted imaging

MRS was analyzed using LCModel (version 6.3). Metabolite concentrations were calculated using water scaling to signal in a water-suppressed reference scan. Data from one mouse in each group were excluded due to poor image and spectrum quality (sham (n=7) and hypoperfused (n=8)). Only values with an estimated standard deviation <25% were used for analysis. Due to this threshold, lactate was analyzed only for a subset of animals (sham (n=5) and hypoperfused (n=6)).

Ventricle to brain ratio (VBR) and hippocampal size were calculated from the T₂ weighted images. Outlines of all structures were manually delineated on a slice by slice basis in ImageJ, and total volumes of each were calculated by multiplying each area by slice thickness (0.45 mm) and summation.

Atlas registration and regional diffusion tensor imaging indices

Two animals, one from each group, were excluded from the analysis due to motion artifact that produced poor quality images (sham (n=9) and hypoperfused (n=10)). Quantitative DTI parameter maps (FA, mean, axial, and radial diffusivities) were calculated using Paravision 6. A mouse brain template (average of 12 different mouse brains), with a corresponding atlas containing masks of 23 different brain regions (modified from MRM NeAT Mouse Brain Database, Stony Brook, USA) was co-registered to the data with a custom Matlab script, which first called the FSL FLIRT tool and second allowed for manual adaption of the transformation in order to improve the procedure. Both the template and atlas were modified manually in ImageJ so that the structures better suited the FA maps in our group of animals. Furthermore, an additional 4 structures were added to the original atlas. A custom ImageJ macro extracted the DTI parameter values across all 23 different brain structures.

Tractography

Subsequent DTI data was analyzed in DSI Studio (http://dsi-studio.labsolver.org). First, tractography data were reconstructed with the DSI model. To select a white matter seed region, a whole brain mask (excluding ventricles) was extracted from the registered atlas. Background voxels with an FA value below 0.6 * the threshold determined by Otsu's method were excluded. Trajectories were subsequently calculated using a 4th order Runge-Kutta method by placing 1 * 10⁶ seeds in the white matter volume (angular threshold 60°, step size half voxel size, minimum/maximum tract length 0.5 mm/20 mm, seed orientation "primary", seed position "subvoxel", no randomization of seeds, direction interpolation "trilinear").

Graph analysis

Graph analysis simplifies DTI data by constructing a graphical representation of nodes (brain structures) and edges (DTI trajectories connecting each structure) in order to obtain numerical values that represent certain network features. This process offers additional information regarding the brain as a whole, and represents an alternative to the qualitative representation of many individual fiber tracts. Adjacency matrices containing the weights of the edges were constructed in DSI Studio for each animal by counting the number of trajectories between the atlas brain structures (23 for each hemisphere). Only tracts that started in one and ended in another region were counted. Matrices were normalized by the total number of seeds and used to calculate graph metrics using the Brain Connectivity Toolbox in Matlab.

Global efficiency is the average inverse shortest path length between all pairs of nodes. Modularity is a measure of the degree to which the overall network can be divided into distinct groups based on a maximum number of within group and minimum number of between group connections. Transitivity and clustering coefficient rely on the presence of triangles (groups of 3
connected nodes). Transitivity expresses the number of triangles as a ratio of the total number of connected triplets. If one particular node is connected to two neighbouring nodes, the clustering coefficient predicts the likelihood that the two neighbouring nodes are also connected and therefore represents the prevalence of clustering around individual nodes (averaged across all nodes). Local efficiency calculates the mean ability of sub-networks around nodes to exchange information when any given node is removed. No significant differences were noted between groups for degrees (a measure of centrality that calculates the average number of nodes that any given node shares a connection with) and assortativity (a measure of resilience that estimates a correlation between the degrees of all nodes on two opposing ends of the network).

**Behavioural testing**

**Novel object recognition**

NOR has been used to examine short term memory by taking advantage of the rodent’s ability to recall features of objects presented a few hours prior; NOR was performed in accordance with a published protocol except testing occurred in a standard mouse housing cage, and the objects were selected according to a different criterion. Different sets of wooden shapes were stored in bags filled with dirty bedding from animals within the same colony. Briefly, a camera was positioned over the test cage in a quiet room. Mice were habituated to the empty cage for 10 min on the day prior to testing. The following day two identical wooden objects were secured in an upright position to the floor of the cage equidistant from the edges. During the first trial, mice were placed inside the cage (snout facing away from the objects) and allowed to explore freely for 10 min. During the second trial (2 hrs later) the left object was replaced with another object of a different shape and smell, and mice were allowed to explore for 5 min. Videos were scored manually and the following parameters were recorded: total time spent with each object (s), total number of visits to each object, and latency to first contact each object (s). The discrimination ratio was calculated using the following formula:  
\[
\text{Discrimination Ratio} = \frac{\text{Time spent with new object}}{\text{Time spent with old object}}
\]

**Water maze**

A standard place task in the Morris water maze was used to assess spatial learning and reference memory. Briefly, a circular maze (120 cm diameter) concealed a submerged escape platform with opaque water. Each animal received three daily trials over 7 consecutive days and the entry point to the pool was randomized with each trial. Trials were spaced approximately 20 min apart, and the animals were kept under a heat lamp in between. Each trial ended when the mouse located the platform, or when the maximum time elapsed (90 s). Animals were collected approximately 10 s after locating the platform. In the case where the maximum time point was reached, animals were guided to the platform and made to wait for 10 s before collection. Escape latency (s), total distance travelled (cm), and swim speed (cm/s) were measured by a computerized tracking system (VideoMot, TSE Systems, Bad Homburg, Germany) and daily data was pooled. At 8 d, a probe trial was performed in which the platform was removed and the mice were allowed to swim for 90 s. The total time spent in the former target quadrant (s) was assessed.

**Tissue preparation and staining procedures**

At the conclusion of the experiments, mice were deeply anaesthetised and perfused through the heart with physiological saline solution followed by 4% paraformaldehyde. Subsequently, the brains were post-fixed for 24 hours, and cryoprotected in 30% sucrose solution before being snap frozen in -40 °C methylbutane. Tissue was sectioned to 30 μm and stored in cryo-protective solution (1 part ethylene glycol, 1 part glycerine and 2 parts phosphate buffered saline (PBS)) at -20 °C.

**Immunohistochemistry**

One series spanning the entire brain from each mouse was processed for standard diaminobenzidine (DAB) immunohistochemistry. Briefly, endogenous peroxidase activity was quenched (10% hydrogen peroxide (30%), 10% methanol, and 80% PBS) for 5 min and blocking was performed for 1 h in Triton-X PBS with 5% normal serum (Vector, Peterborough, UK). Tissue was rinsed extensively between steps with PBS. Sections were incubated with 1:2000 rabbit anti GFAP (Dako, Hamburg, Germany) and 1:500 rabbit anti Iba1 (Wako, Neuss, Germany) in Triton-X PBS overnight at 4 °C. The following day, the corresponding biotinylated secondary antibodies (1:100) were applied for 2 hours at room temperature, followed by a 1 h incubation in avidin and biotinylated horseradish peroxidase (Vectastain Elite ABC kit, Vector, Peterborough, UK). Visualization was performed with DAB (DAB Substrate kit, Vector, Peterborough, UK).

**Luxol fast blue**

One series spanning the entire brain from each mouse was mounted on slides and air-dried overnight. The following day, slides were placed in 70% ethanol for 30min followed by incubation in Luxol solution (2% in 96% ethanol with 0.5% acetic acid (10% stock solution)) at 60°C for 1:15 h. Sections were rinsed quickly in distilled water followed by a dip in 0.05% Li2CO3 to differentiate the white matter and a series of ethanol rinses (10 s in 95%, 1 min in 96%, and 30 s in 100%). Slides were cleared with Rotihistol and coverslipped with Vitro-Clud (R. Langenbrinck Labor und Medizintechnik, Emmendingen, Germany).

**Statistical analysis**

Normal distribution and homogeneity of variance were established with the Kolmogorov-Smirnov and Levine’s tests, respectively, using SPSS Version 22 software (IBM, Hampshire, UK). CBF data, escape latencies in the water maze, and discrimination ratios in the NOR test were compared using a mixed design two way repeated measures ANOVA with surgery (sham versus microcoil) as the between subject factor, and time as the within subject factor. Performance in the water maze
probe trial, vascular size, VBR, and hippocampal size were compared between groups using an un-paired Student’s t-test. Spectroscopy data, DTI parameter maps, and network analysis metrics were compared between groups using a series of un-paired Student’s t-tests followed by a False Discovery Rate Control for multiple comparisons and Type I errors. All reported p values have been corrected. Correlations between performance in the water maze (area under the curve over the course of the 7 test days) and network analysis parameters were performed using Pearson’s correlation. The logistic regression model for the graph theory data was performed in R Version 3.3 software (Free Software Foundation, R Foundation).

Supplemental figures and figure legends

Figure I. Morphological changes in the hypoperfused brain. (A) Overall size of the Circle of Willis vasculature (means ± SD) in sham (n=10) and hypoperfused (n=11) groups, and (B) Ventricle to brain ratio (VBR).

Figure II. Graph theory revealed microstructural network changes in the hypoperfused brain. (A) Clustering coefficient, (B) Degrees, and (C) Assortativity in both groups.
Figure III. The effects of hypoperfusion on distance travelled and reference memory in the Morris water maze. (A) Total distance travelled during the place task, and (B) time spent in the target quadrant during the probe trial (means ± SD).

Figure IV. Microcoil manufacture influences material integrity. Photographs of microcoils harvested at the conclusion of the experiments from a sham (A) and hypoperfused (B) animal (500, and 160 µm diameter microcoils, respectively, Shannon, Coiled Springs Microcoil, Limerick, Ireland) show that the carotid arteries were still constricted at the end of the experiment in the hypoperfused group. Microcoils harvested from a hypoperfused animal (C) implanted with 180 µm diameter coils (from Sawane Spring Company, Hamamatsu, Japan) show substantial degradation after 1 month in the body.

Figure V. Titrated cerebral blood flow (CBF) in the differentially hypoperfused brain. (A) CBF over time (means ± SD) in the sham (n=10) and hypoperfused (n=11) groups (500 and 160 µm diameter microcoils, respectively) with data superimposed from a group that was hypoperfused with standard 180 µm (n=5) diameter coils.
Supplemental References


## Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
</tr>
</thead>
</table>
| **Experimental groups and study timeline** | ✓ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.  
✓ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated.  
✓ An overall study timeline is provided. |
| **Inclusion and exclusion criteria** | ✓ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article. |
| **Randomization** | ✓ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided.  
✓ Type and methods of randomization have been described.  
✓ Methods used for allocation concealment have been reported. |
| **Blinding** | ✓ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible.  
✓ Blinding procedures have been described with regard to masking of group assignment during outcome assessment. |
| **Sample size and power calculations** | ✓ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided. |
| **Data reporting and statistical methods** | ✓ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups.  
✓ Baseline data on assessed outcome(s) for all experimental groups have been reported.  
✓ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms.  
✓ Statistical methods used have been reported.  
✓ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures. |
| **Experimental details, ethics, and funding statements** | ✓ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described.  
✓ Different sex animals have been used. If not, the reason/justification is provided.  
✓ Statements on approval by ethics boards and ethical conduct of studies have been provided.  
✓ Statements on funding and conflicts of interests have been provided. |