Supplemental material

Hypoxia induces \textit{de novo} formation of pial collaterals and lessens the severity of ischemic stroke

Hua Zhang, Wojciech Rzechorzek, Amir Aghajanian, James E. Faber

1. Detailed materials and methods.
2. References
3. Supplemental data:
   - Supplemental table I. Target genes and TaqMan assay ID numbers for quantitative RT-PCR.
   - Supplemental table II. Infarct volume data for Figure 3
   - Supplemental figure I. Measurement of CO$_2$ levels in hypoxia chamber.
   - Supplemental figure II. Conditional knockdown efficiencies.
   - Supplemental figure III. Cumulative frequency distribution plots of collateral diameters.
Materials and Methods

Animals. C57BL/6J (B6) mice were from the authors’ laboratory colony that is rejuvenated at 2-year intervals with breeders from Jackson Laboratories. Rabep2\(^{−/−}\) knockout colony mice were created by crossing heterozygous B6.Cg-Tg (CAG-Cre/Esr1) 5Am/j (referred to here as CAG-Cre\(^{ERT}\)) mice (JAX #004682) to B6.Cxc4\(^{−/−}\) (JAX #008767, B6;129P2-Cxc4\(^{−/−}\) mice) or B6.Vegfa\(^{−/−}\) (gift from Genentech) to create B6.CAG-Cre\(^{ERT+}\);Cxc4\(^{−/−}\) and B6.CAG-Cre\(^{ERT+}\);Cxc4\(^{−/−}\);Vegfa\(^{−/−}\) littermates or B6.CAG-Cre\(^{ERT+}\);Vegfa\(^{−/−}\) and B6.CAG-Cre\(^{ERT+/−}\);Vegfa\(^{−/−}\) littermates. Endothelial cell (EC)-specific inducible Flk1 knockdown mice were created by crossing B6.Cdh5(PAC)-Cre-ERT\(^{T2}\) mice (gift from Ralf Adams) to B6;CD1-Flk1\(^{−/−}\) (gift from Jody Haige, back-crossed to B6 for more than 10 generations) to create B6.Cdh5(PAC)-Cre-ERT\(^{T2+}\);Flk1\(^{−/−}\) and Cdh5(PAC)-Cre-ERT\(^{T2+}\);Flk1\(^{−/−}\) littermates. Inducible Cre activity was activated by ip injection of tamoxifen (T5648, Sigma). Tamoxifen was dissolved in 100% ethanol, diluted 1:9 with filtered corn oil, and sonicated (final concentration 40mg/ml). For the Figure 5 experiments, each mouse was injected with 100ul/30 gram body weight (4mg/30g) for 3 consecutive days before the first day of acclimation at 16% FIO\(_2\) (followed by 14, then 12% on consecutive days, the latter beginning the 4 week period of hypoxia (Hx)). A booster injection of tamoxifen was given (4mg/30g) every 7 days after the Hx period commenced. Knockdown efficiency was measured by qRTPCR in neocortex collected immediately after mice were removed from Hx to minimize any decline in mRNA for Vegfa and other genes that decline rapidly in normoxia (Nx).

Both sexes were studied in ~equal numbers in each experiment, however n-sizes were not powered to test for sexual dimorphism. In a previous study, no sex-dependent differences were observed for native pial collateral number or diameter.\(^3\) All mice were 2.5-5 months-age at the time of onset of acclimation to Hx or exposure to the indicated level of hypoxia. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC# 18-123.0-A, 04/2019) and were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the ARRIVE and STAIR guidelines.

Hypoxia. Four hypoxia levels were examined: 7, 8.5, 10 and 12% FIO\(_2\). For 7% FIO\(_2\), oxygen was lowered 1%/day over 2 weeks during acclimation, then maintain at 7% FIO\(_2\) for 2 weeks, followed by recovery to normoxia (Figure 1A). For 8.5% FIO\(_2\), oxygen was lowered 2%/day during acclimation. For 10% FIO\(_2\), oxygen was lowered to 16% for 1 day, then 12% for the next 2 days, then maintain at 10% for the indicated number of weeks. For 12% FIO\(_2\), oxygen was lowered to 16% for 1 day, then 14% for the next 2 days, then maintained at 12% for the indicated number of weeks. We used two hypoxia chambers (Oxycycler, Biospherix Ltd; and one custom made). Both chambers are of similar internal dimensions (76x51x51 cm and 60x55x30 cm, respectively) and hold 4 cages (each filter-topped cage was 27x16x13 cm) with 4-5 mice/cage). Inflow rate (nitrogen mixed with oxygen or room air, depending on the above two chambers) was ~3.12 ft\(^3\)/h (1 nitrogen tank, NF300 AirGas, lasted 3-4 days at 10% FIO\(_2\)), and holes (0.7 cm diameter) were opened at the bottom of each of the Biospherix’s 3 sides (4 holes) and the custom chamber’s 4 sides (1 hole). Drierite (#21909-5000, Acros, Fair Lawn, NJ) and soda lime (#36596, Alfa Aesar, Haverhill, MA) were placed at the bottom of the chamber in 12x10x5 cm trays (~260g and 200g, respectively). Drierite was changed when bedding was changed (every 5-7 days), soda lime with every experiment. The hypoxia chambers have internal fans to prevent gradients in composition of the atmospheres. An experiment was conducted to ascertain CO\(_2\) levels (Supplemental figure I). A CO\(_2\) sensor (GC-0006, CO2Meter Inc, Ormond Beach, FL) was placed in the Biospherix chamber at the bottom of one of the 4 cages (each with 4 mice) at mouse-head level when sitting, and surrounded by a protective sheath. CO\(_2\) was automatically recorded at 5 min intervals. FIO\(_2\) was lowered from 21% to 10% in ~3 h. CO\(_2\) was recorded at 5 min intervals for one week without opening the chamber. CO\(_2\) level varied with the awake/activity state of the animals. Lights were on for 0700-1900. CO\(_2\) in the hypoxia chamber (Supplemental figure I) remained well below levels that: 1) when inspired for 1 month cause mild respiratory stimulation (≥1.5% FICO\(_2\)), 2) lessen intracerebral angiogenesis induced by 4 weeks of
10% FIO₂ (6.5-7% FICO₂), and 3) modify the pattern of expression of angiogenic genes (50% FIO₂, 2.5% FICO₂).³⁷

**Angiography and morphometry.** As previously described in detail⁸ animals were anesthetized deeply with ketamine and xylazine, and heparinized. The distal thoracic aorta was cannulated, right atrium perforated, and the mouse was perfused with PBS containing sodium nitroprusside (10⁻⁴M, for maximal dilation) and Evan’s blue dye (for light staining of brain and the endothelial surface) at ~100 mmHg. Yellow Microfil (Flowtech Inc, Carver, MA) was infused at a viscosity adjusted to fill the entire pial arterial and collateral circulations with sufficient pressure (~100 mmHg) and duration to cause limited capillary transit and venous filling to assure complete filling of all precapillary vessels. After the Microfil had set, brains were kept in 4% PFA and collaterals were imaged the next day using a Leica fluorescent stereomicroscope. All collaterals between the anterior cerebral artery (ACA) and middle cerebral artery (MCA) trees of both hemispheres were counted, as were all intra-tree anastomoses (ITAs) present in both MCA trees. Images were subsequently analyzed (ImageJ, NIH): Collateral lumen diameter was determined at midpoint at 50X and averaged for each mouse. We counted the number of pial branches (of both hemispheres, averaged) that branched from the largest second-order branch of the MCA tree (M2-MCA), including small branches from it that became penetrating arterioles, extending from its bifurcation from the M1-MCA to the watershed/border zone; the lumen diameters of the large branches (M2, M3, etc) were determined at the midpoint along the length of each successive branch-order beyond their point of bifurcation from the parent vessel. Lumen diameters of the vessels of the circle of Willis were determined as described previously.⁹

**Permanent middle cerebral artery occlusion (pMCAO) and determination of infarct volume and hematocrit.** As previously described,⁸ mice were anesthetized with ketamine and xylazine (100 and 10mg/kg, ip, respectively) and rectal temperature was maintained at 37±0.5°C. The temporalis muscle between the right eye and ear was retracted along a 4 mm skin incision. The oblique edge of a 2.1 mm drill bit (19007-21, FST, Foster City, CA) was used to thin an approximately 1 mm circle of bone overlying the distal M1-MCA. The thinned bone and dura matter were incised with a 27-gauge needle tip and reflected to expose the distal M1-MCA. The latter was cauterized (18010-00, FST, Foster City, CA, modified tip) just distal to the lenticulostriate branches. The incision was closed with suture (~15 min total surgery time), intramuscular cefazolin (50 mg/kg) and buprenorphine (0.1 mg/kg and again 12 h later) were administered, and the animal was monitored in a warmed cage during recovery from anesthesia to maintain rectal temperature. Mice were euthanized 24 hours after pMCAO. Brains were sliced into 1 mm coronal sections and incubated in 1% 2,3,5-triphenyltetrazolium chloride in PBS at 37°C. Left and right forebrain hemispheres and infarcted tissue in the right hemisphere were imaged on both sides of each slice with a stereomicroscope using ImageJ software (NIH), average areas were determined for each slice, and tissue volumes were calculated. Percent infarct volume was normalized to forebrain volume: infarct volume = the sum of the (lesion area divided by total forebrain area) x 100] determined for both sides of each of 7 slices, multiplied by slice thickness (see Supplemental figure II).

Hematocrit values shown in Figure 3C were measured from blood taken via retro-orbital venipuncture during normoxia and on day 35 for 7% FIO₂ group and on the day of removal from hypoxia for the other groups. In the “hematocrit corrected” group shown in Figure 3D hypoxic polycythemia was lessened (as much as possible while permitting survival -- determined in pilot experiments) by removing 0.5-0.6 ml (0.53 ± 0.05 ml, n=7) of blood via retro-orbital venipuncture on the day of removal from hypoxia, followed by infusion of 0.6 ml of sterile mouse plasma (BioIVT, #Mseplnahp-C57). Hematocrit determined 5 days later was 51.7 ± 3.8%. In the latter group pMCAO was then performed, and infarct volume was measured 24h later.

**Quantitative RT-PCR (Figure 4 and Supplemental figure II).** C57BL/6 mice were acclimated to 1 day of 16% FIO₂, then 2 days at 12% FIO₂, followed by exposure to 10% FIO₂ for 1, 7 or 14 days whereupon the mice were removed from the Hx chamber and the neocortex rapidly removed to liquid nitrogen. Total RNA was isolated using a combination of TRIzol™ lysis (ThermoFisher, #15596026)
and the Qiagen mini-RNA kit according to manufacturers’ instructions. RNA concentration and quality were determined using NanoDrop (ND-1000). Reverse transcription was performed with SuperScript™ III First-Strand Synthesis System (ThermoFisher, #11752-050) following the manufacturer’s instructions. Amplification was achieved with TaqMan Fast Advanced Master Mix (ThermoFisher, #4444556) on StepOnePlus (AB Biosciences) and analyzed using the delta-delta CT method and StepOnePlus software. Samples were analyzed in triplicate. The target genes and their Taqman assay ID numbers are listed in Supplemental table I.

**EdU labeling of pial collaterals.** Male and female 10 week-old C57BL/6 mice were studied. Mice were exposed to 16% FIO₂ on day-1, 14% on day-2 and -3, then maintained at 12% FIO₂ for 6 weeks. EdU (Click-iT™ Plus EdU Cell Proliferation kit (Cat # C10638, ThermoFisher) was dissolved in saline (2 mg/ml) and 1mg was injected i.p. per mouse on day-6, -10, -18, -26, -34 and -42. Mice maintained in normoxia received EdU on the same days. Thereafter, mice were anesthetized and perfused with PBS containing sodium nitroprusside (10⁴M, for maximal dilation) and perfusion fixed with 1% PFA for 5 min. The dorsal surface of the neocortex of both hemispheres was frozen onto dry ice-cold glass slides and shaved down to ~1mm thickness, then treated with 0.5% Triton X-100 and 3% BSA for 30 min at room temperature. EdU staining was according to the manufacturer with modification of staining time to 2 hr at room temperature and overnight at 4°C. Endothelial cells of pial vessels were stained with DyLight 488 lycopersicon esculentu and non-endothelial cells based on the former’s nuclear orientation with the vessel axis. Fluorophores were excited at 568nm (Alexa Fluor 555-conjugated to EdU) and 460nm (Hoechst 33342).

**Statistics.** Experiments were performed in accordance with the ARRIVE guidelines, and the following suggested STAIR criteria:10,11 n-sizes (number of animals) were specified based on our previous studies8-12,8,9 demonstrating sufficient power to test the endpoints measured herein; approximately equal number of males and females were included; investigators were blinded during data analysis where possible; the review, discussion and citation of the literature was unbiased; MCAO was used to permanently recruit blood flow across pial collaterals. Values are mean ± SD. Statistical analysis (p<0.05 = significant) was as follows (the figure legends also give the individual tests. 17 experiments were conducted in the study (~500 mice). Data from each of the 227 bars presented in Figures 1-7 and Supplemental figure II, except for those whose nature/formats do not permit testing for normality (figures 2A, 6C and Supplemental figures I and IIIA-D), and that had n ≥ 7 (61 bars had n < 7), which is required for normality testing (D’Agostino-Pearson test, Graphpad Prism), were normally distributed (p ≥ 0.05) except for 5 bars: Figure 1F bar4 p=0.031; Figure 2B bar1 p=0.014, bar17 p=0.027; bar21 p=0.0003; Figure 5 middle-right panel p=0.0002. Data in the figures are presented as dot blots, except for figure 2A and Supplemental figures I and IIIA-D whose formats did not permit this to be done.

Figures 1,3,5,6, Supplemental figure II: pre-specified single or multiple Bonferroni protected 1-sided Students’ t-tests, alone or after 1-way ANOVA, because our literature-based a priori hypothesis was that hypoxia would have no effect or increase the endpoints in Figure 1, Figure 3A-C, and Figures 5,6, or decrease the endpoint in 3D. Figure 2A, Chi-squared tests. Figure 2B,C: pre-specified 2-sided Students’ t-tests because our literature-based a priori hypothesis was that hypoxia would have no effect, increase, or decrease gene expression; 2-sided Students’ t-tests versus normoxia because biphasic responses for one or more of the genes was expected a priori, and because the 3 time points were pre-specified as independent null hypotheses (ie, no difference in expression compared to normoxia).

As stated in Methods, Supplemental Methods (above) and in the figure legends, the statistical tests of the data in each figure’s experiments were pre-planned for: 1) test to be used, 2) post-test number of Bonferroni comparisons, and 3) p-value sidedness. This was done before each experiment so as to
confine the testing to the specific questions that addressed the specific hypotheses, which were stated in the text and/or implied by the comparisons that were done. Comparing all bars in all directions is invalid. For example, in Figures 1 and 2 we did not test whether normoxia-Rabep2−/− differed from normoxia-WT in a given panel or bar-grouping because we had already examined that question in previous studies10,29 described and referenced in the Introduction, Results or Discussion. As another example, the white bar comparisons in Fig 6D were previously tested and discussed in Introduction,10 thus were not pre-specified to be tested again in the present study. As well, in Figures 1 and 2 and elsewhere, the hypoxia group was pre-planned to only be compared to its immediate control group so as to provide the most specific comparison. We did not inspect the findings of any experiment and then decide what comparisons to test. Regarding the experiments in Figure 3, only certain of the experiments in the figure were pre-specified for 1-way ANOVA. These experiments are indicated by the horizontal brackets above the bars, above which the p value of the F statistic is given; the other groups were pre-specified t-tests.

References
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**Supplemental table I.** Genes and TaqMan assay ID numbers for quantitative RT-PCR

| Target gene | Assay_ID               |
|-------------|------------------------|
| 18s/45s     | Mm03928990_g1          |
| Act-B       | Mm02619580_g1          |
| Tie2        | Mm00443243_m1          |
| Ang2        | Mm00657574_s1          |
| CXCR4       | Mm01996749_s1          |
| Hif1a       | Mm00468869_m1          |
| Hif2a       | Mm01236112_m1          |
| Rabep2      | Mm00518884_m1          |
| VEGF-A      | Mm00437306_m1          |
| Flk1        | Mm01222421_m1          |
Supplemental table II. Infarct volume data for Figure 3

| Experimental group                                      | Body wt at harvest, g | p value 2-tail | Forebrain volume, mm³ | p value 2-tail | Forebrain vol/b wt | p value 2-tail |
|---------------------------------------------------------|-----------------------|----------------|------------------------|----------------|--------------------|----------------|
| Normoxia, n=10                                         | 23.5±2.2              |                | 367.1±23.2             |                | 15.8±1.7           |                |
| 10% FIO2 4w, HCT corrected, n=7                         | 26.3±3.4              | 0.05           | 354.4±22.2             | 0.277          | 13.7±1.9           | 0.04           |
| 10% FIO2 4w, HCT not corrected, n=8                     | 23.4±1.9              | 0.93           | 367.3±22.5             | 0.982          | 15.8±1.2           | 0.99           |
| 10% FIO2 4w, then Nx 6w, n=8                           | 26.8±4.5              | 0.05           | 405.8±25.0             | 0.004          | 15.4±2.0           | 0.69           |
| 12% FIO2 8w, then Nx 6w, n=8                           | 25.5±3.5              | 0.19           | 339.0±20.1             | 0.003          | 13.6±2.6           | 0.06           |

| Infarct vol, mm³ | p value 1-tail | Infarct vol/ body weight | p value 1-tail | Inf vol, % forebrain | p value 1-tail |
|------------------|----------------|--------------------------|----------------|----------------------|----------------|
| Normoxia, n=10   | 15.1           | 0.66±0.34                |                | 4.13                 |                |
| 10% FIO2 4w, HCT corrected, n=7                         | 7.4             | 0.02                     | 0.28±0.24       | 0.012               | 2.07           | 0.02           |
| 10% FIO2 4w, HCT not corrected, n=8                      | 14.1            | 0.40                     | 0.60±0.39       | 0.270               | 3.84           | 0.07           |
| 10% FIO2 4w, then Nx 6w, n=8                             | 9.4             | 0.08                     | 0.33±0.28       | 0.020               | 2.31           | 0.04           |
| 12% FIO2 8w, then Nx 6w, n=8                             | 7.3             | 0.01                     | 0.27±0.28       | 0.050               | 2.23           | 0.03           |

Infarct volume was normalized to body weight and to percent of forebrain volume (the latter given in Figure 3) to account for variation in body weight. Variation in body weight among the experimental groups likely reflects differences in age (3.6-6.5 months) and sex (~equal number for each group). Collateral number and diameter are comparable for males and females² and for the above age-range.¹² Statistical comparisons and tailedness were pre-specified according to the hypotheses described in Introduction and Results.
Supplemental figure I. CO₂ levels in hypoxia chamber varied with activity state of the animals during six days of exposure to 10% FIO₂. Hypoxia chamber contained 4 cages with 4 mice in each cage (this same caging was used in all experiments) and was maintained at 10% FIO₂. A CO₂ sensor was placed in one of the cages ~1 cm above the bedding. CO₂ was recorded every 5 minutes for 6 days without opening the chamber. CO₂ and humidity absorbents (soda lime and Drierite; see Methods) and bedding were changed between 0900-0100h on day 7, resulting in the nadir for CO₂ subsequently returning to ~0.2% (data not shown); this same schedule was used in all experiments. CO₂ measured outside of the chamber was ~0.04%, in agreement with the mean value of 406 ppm reported by NOAA for October, 2018. Lights were on 0700-1900h. Small peaks at ~1400h on day 2 and 3, as well as the early first-peak on day 5, presumably reflect increased activity of the mice caused by human activity in the vivarium room where the chamber was housed. FICO₂ remained below levels that: 1) when inspired for 1 month cause mild respiratory stimulation (≥1.5% FICO₂), 2) lessen intracerebral angiogenesis induced by 4 weeks of 10% FIO₂ (6.5-7% FICO₂), and 3) modify the pattern of expression of angiogenic genes (50% FIO₂, 2.5% FICO₂). See text for refs.
Supplemental figure II. qPCR assessment of efficiency of knockdown of Vegfa, Flk1, and Cxcr4 in experiments shown in Figure 5. Neo-cortex was frozen immediately after removing mice from the hypoxia chamber on day-7 of exposure to hypoxia, n=3-4. *,**, p<0.05, 0.01 vs. normoxia. #,## p<0.05, 0.01 vs. second bar in panel. Percentages given in black bars are relative to their paired white bar. Percentages given above bars are relative to their values in their same-color CreERT2 bars.
Supplemental figure III. Cumulative frequency distribution plots of collateral diameters for mice in the group defined in the key for each graph. IIIA, Data obtained from mice in groups shown in Figures 1 and 3. IIIA, 12% FIO₂ experiment. IIIB, IIIC, IID (see continuation pages below), 10% FIO₂, 7% FIO₂, and Rabep²⁻ experiments, respectively. Two modes (local maxima) are not evident in the middle plot of IIIA, middle plot of IIIB, or second plot of IIIC or IID. See text of Results for further discussion.
Supplemental figure IIIB.
Supplemental figure IIIC

Supplemental figure IIID