## Methodologies are described in detail below for the three main experiments conducted: 1) Memory Testing with anemia, 2) Cerebral Oxygenation with anemia, and 3) Molecular Markers of Hypoxia with anemia.

## **Approvals**

## Approval from the Institutional Animal Care and Use Committee at the University of Pennsylvania was obtained prior to the conduct of this study.

***Euthanasia***

All animals were euthanized using a University Laboratory Animal Resource accepted protocol.

## **Animals and Experimental Groups**

We have chosen to conduct this series of experiments in the SHR 1 model for the many similarities between this strain and humans in hypertensive cerebrovascular disease. Hypertension is usually developing by age 4 months and well established by 6 months of age. Hypertension related changes in the brain microvasculature of this strain are apparent by 6 months of age2,3. Cerebral cortical, forebrain, and hippocampus changes include decreases in nerve cell number, increase in the number of glial fibrillary acidic protein immunoreactive astrocytes, and increased occurrence of apoptosis in older SHR animals 4,5 in comparison to the nonhypertensive Wistar-Kyoto strain. Aging in this strain increases vulnerability to cerebral ischemic insults6. Mortality in the SHR is approximately 50% at 18 months 7.

## Three month old SHR were obtained (Harlan Sprague Dawley Inc., Frederick, MD) to create the young (Y) groups. Retired male breeders, also obtained from Harlan Sprague Dawley Inc. at 9-12 months of age, were used to create the mature groups (M), and or housed in the animal facility until appropriately aged (A), 13-18 months. Animals were housed 2-3 rats/cage and acclimated for at least 2 weeks prior to testing. Animals were handled frequently during acclimatization. During this period of time animals were allowed ad libitum diets.

Within the three age groups, Y, M, and A, two treatment subgroups within each were included. The anemic subgroups are referred to as YAn, MAn, AAn underwent the true hemodilution protocol, while animals undergoing the sham isovolemic protocol are referred to as YSh, MSh, and ASh.

## **Anesthetic Management**

For Memory Testing and Molecular Markers of Hypoxia experiments, rats were anesthetized in a plastic box containing 4% isoflurane in 100% oxygen. A nose cone with spontaneous ventilation was used for the maintenance of anesthesia utilizing 1.5– 2.0% isoflurane in 21% oxygen. Surgery was performed with animals supine on a TC-1000 Rat thermistor controlled warming blanket (CWE, Inc., Ardmore, PA.)

For Cerebral Oxygenation, rats were anesthetized in a plastic box containing 4% isoflurane and 100% oxygen, tracheotomized with a 14-guage catheter, and ventilated with a tidal volume of 10mL/kg, at a rate of 40-50 breathes/minute, and utilizing a volume control rodent ventilator (CWE, Inc., model MRI-1). Anesthesia was maintained with 1.5-2% isoflurane in 21% oxygen and alpha-chloralose (80mg/kg) injected into the axillary fat pad prior to craniotomy. The anesthetic α-chloralose was utilized in this experiment as it has minimal effects on cerebrovascular reactivity as compared to its volatile anesthetic counterparts8. Anesthetic management did not include muscle relaxants.

***Hemodilution and Sham Hemodilution Protocols***

For Memory, Cerebral Oxygenation, and Molecular Markers of Hypoxia experiments, under sterile conditions, an incision over the right groin was made. The right femoral vein was cannulated with polyethylene-10 tubing that was fixed in place with suture for the hemodilution process and for blood sampling for hemoglobin and hematocrit. The isovolemic hemodilution process involved three weight-based withdrawals of 2-5 ml (7 ml/kg) of venous blood. For YAn, MAn, and AAn groups, the blood removed was immediately replaced with warmed normal saline at 37°C in a ratio of 1: 3 (blood: normal saline). For sham groups (YSh, MSh, and ASh), withdrawn blood was immediately re-injected. The hemodilution protocol was designed to decrease the hemoglobin concentration in a step-wise fashion from a baseline of ≈ 12 g/dl to a final level of ≈ 5 g/dl after the third hemodilution. Hematocrit and hemoglobin were measured after each hemodilution utilizing a Stat Profile pHOx system (Nova Biomedical, Waltham, MA). The sham hemodilution groups allowed us to control for anesthetic exposure, hypotension related to experimental hemorrhage, surgical stress, and wound related disability.

For animals in the Memory Testing and Molecular Markers of Hypoxia experiments, after hemodilution was completed, the groin incision was closed with absorbable suture and the anesthetic discontinued. Animals were recovered under warming lights in cages in the laboratory. When animals demonstrated normal ambulation they were returned to the animal facility.

## **Visuospatial & Reference Memory Testing**

The water maze tank utilized measured 200 cm in diameter and 60 cm in height, and was filled with water that was made opaque with nontoxic titanium dioxide powder and maintained at the temperature of 22 ± 2°C. The water level in the tank was 25 cm high, which was 1.5 cm above the escape platform. The escape platform was made of transparent plastic, 20 cm in diameter, 23.5 cm in height, and had a rough surface for the animals to grip on to when climbing on the platform.

The water maze paradigm used in this analysis is one described by Chen et. al.9, and is a variant of the repeated acquisition, moving platform test. This paradigm is complicated in that previously learned platform locations may result in interference for the learning of new platform locations. Learning in such a paradigm may demand elements of both longer-term procedural memory as well as processing of acutely presented spatial information. Details are discussed below and the timeline for training and testing can be found in the main manuscript, Figure 1.

*Pre-hemodilution flagged (cued) platform training.* During initial training, all rats were trained to swim to the submerged platform marked with a vertical flag. Training consisted of 4 trials per day, for 5 days, (main manuscript, Figure 1, days 1–5). During each trial animals were allowed up to 90 s to find the platform, after which they were then permitted to sit for 15 s before being removed from the pool. If an animal did not find the platform within 90 s, it was gently guided to the platform and allowed to again remain there for 30 s. The platform was placed in the same target quadrant for all 20 cued trials over the 5 day period. For each trial the rat was placed into the water facing the wall of the tank, using a randomly selected starting point. A white curtain surrounded the pool to prevent confounding visual cues. Between trials, animals were placed in a holding cage, under a heat lamp until dry.

*Pre-hemodilution non-cued platform training & reference memory testing.* Following two days of rest after the 5 day cued training period, pre-hemodilution non-cued training was performed for 4 consecutive days (main manuscript, Figure 1, days 8–11). During this period animals were trained to access a submerged, non-flagged platform using fixed accessory visual cues on the walls and ceiling surrounding the water tank. The platform was centered in the same target quadrant (P-1) for all trials during this 4 day training period, but the starting points were again random for each rat. In order to reach training criterion, the rats were required to reach the platform within 90 s and were allowed a maximum of 4 trials per day. As previously described, animals were allowed to sit on the platform for 15 s if acquired within the allowed time frame, and rats, when failing a trial, were gently guided to the platform and allowed to remain for 30 s.

On day-12, baseline, pre-hemodilution reference memory performance was tested using the same platform locations as had been trained upon in the previous four days. *Escape* *Latency*, *Swim Speed, and Distance* were recorded for each of 4 trials. For any trial where the animal did not reach the platform within 90 s, latency was recorded as 90 s. Also on Day-12, following completion of the 4th trial, a single *Probe* trial was performed to further document baseline reference memory (main manuscript, Figure 1, Day 12). For this trial, the platform was removed from the water maze and the movements of the rat were recorded for 90 s. The time and percentage of time spent in each quadrant along with the number of platform crossings were recorded.

*Post-hemodilution reference memory testing.* The first day of testing following hemodilution, Day-17, the reference memory paradigm conducted on Day-12 was repeated and was conducted on the same platform location (P-1) as the rats had been trained upon in the previous week. This allowed for a more gradual reintroduction to the memory task and allowed an assessment of the impact of the interventions upon reference memory, prior to testing on the more challenging moving platform working memory paradigm on days 18-26.

*Post-hemodilution working memory and learning testing.* Beginning on Day-18, rats underwent 9 days of testing using a moving platform paradigm (main manuscript, Figure 1, Days 18–26). Animals were again allowed 90 seconds to find the hidden platform during each trial. If the animal was not successful, it was guided to the platform and was allowed to stay there for 15 seconds. For these trials, criteria for advancing to a new platform position, on the next day, was reaching the platform position in less than 15 s, for 3 consecutive trials. Once criterion was reached, testing for that animal, on that platform, and on that day was ended. If the criteria was not reached within 8 trials, then the rat would again be tested the next day with the same platform location and with 8 trials per day, until criteria was reached. For these trials, the time to reach the platform, the number of trials, and the swim speed were recorded.

*Definition of Water Maze Measurements.*

Reference Memory Testing

1 ) Escape Latency (s) - the time between leaving the starting point and climbing onto the escape platform.

2) Probe Testing

a) Distance (cm) –the distance traveled from starting point to platform escape.

b) Platform Crossings (#) - the number of crossings over the escape platform location.

c) Quadrant Time Percent (%) - the percentage of time spent in the quadrant of the escape platform.

Working Memory and Learning Testing

1) Trials to Criteria (TTC) (#) - the number of trials that the rat required to reach changing platform memory criteria.

## All water maze trials were recorded via the IMAQ video camera and control system (National Instruments, Austin, TX) that was mounted above the center of the tank. Actimetrics (WM Actimetrics, Evanston, IL) water maze tracking software was used to store and export data.

***Cerebral Oxygenation***

Under sterile conditions, both femoral arteries were cannulated with polyethylene-50 tubing, the right femoral artery for continuous blood pressure measurement and the left for arterial blood gas measurements and hemodilution. The rat’s head was stabilized in a stereotaxic frame and a midline scalp incision performed. The scalp over the fronto-parietal cortex was retracted, and the skull was cleaned.

A 4 x 6 mm flap was removed and the dura carefully incised and retracted. Following dural retraction, a 450 μm diameter Optronix multi-parameter probe (Oxford Optronix, Oxford, United Kingdom), was positioned over a point 4 mm posterior to Bregma and 4 mm lateral to the sagittal suture. The multi-parameter probe was used to measure laser Doppler flow, partial pressure of brain oxygen, and brain tissue temperature. The probe was stereotactically placed using a micromanipulator, 2-4 mm into the parietal association cerebral cortex, under direct vision, at a magnification of 6X. Care was taken to avoid placing the probe into or next to large pial vessels. Isoflurane was discontinued following craniotomy and a period of 45 minutes was allowed to pass to allow for the elimination of isoflurane and for stabilization of the brain tissue prior to data collection.

The Optronix tissue oxygen probe employs fluorescence quenching from a ruthenium-based fluorophore. Sampling volumes are thought to be approximately 0.25-0.35mm3. The most sensitive O2 measurements occur over the range of 0-60 mmHg. LDF sampling volumes are estimated at 0.3 -0.5 mm3. Laser Doppler flow, brain oxygen, and brain temperature were monitored using OXYLAB multi-parameter monitors (Oxford Optronix, Inc.). Mean arterial pressure was monitored utilizing an ADI invasive pressure transducer interfaced with an ADI blood pressure amplifier (ADInstruments Pty Ltd, Castle Hill, NSW, Australia). Rectal temperature was monitored via probe and maintained with a CWE thermo-regulated heating pad (CWE, Inc.), set at 37 ± 0.5 C.

After each of the three hemodilution steps, a period of 10 minutes was allowed for hemodynamic stabilization. The arterial blood samples were analyzed utilizing a Stat Profile pH Ox system (Nova Biomedical, Inc.). Measurements of pH, arterial partial pressure of carbon dioxide, arterial partial pressure of oxygen, hemoglobin (g/dl), and oxygen content (ml/dl) were obtained. Ventilation was adjusted to maintain arterial carbon dioxide within the range of 38-42 mm-Hg.

Following the initial 45 minute (following probe placement) and subsequent 10 minute stabilization periods (following each of the three hemodilution steps), all measured physiologic parameters were averaged over a 5 minute time period. Thus, data from four time points was recorded: Time 0-baseline prior to hemodilution, Time 1-following hemodilution #1, Time 2-following hemodilution #2, and Time 3-following hemodilution #3.

All physiologic measures were recorded simultaneously and continuously for the duration of the experiment. These data were integrated through an 8 channel ADI PowerLab acquisition system. Continuous digital recording of data was accomplished through interfacing the ADI PowerLab system with Chart 5.0 software interface (ADInstruments Pty Ltd.) on a Power Mac computer (Apple Computer, Inc., Cupertino, CA.). All analogue data were sampled digitally at a frequency of 10 Hz.

***Molecular Markers of Hypoxia***

*Quantitative Real-Time Polymerase Chain Reaction*

Under 1.5-2% isoflurane in air, three groups of SHR, Young (Y), Mature (M), and Aged (A) underwent isovolemic (An) or sham (Sh) hemodilution protocols. At 24 or 48 hours following hemodilution, animals were sacrificed, brains extracted, and rapidly dissected on ice to recover cortex and hippocampus, which were then placed in Trizol medium prior to flash freezing in liquid nitrogen and storage at -80°C. Total ribonucleic acid was extracted from rat hippocampus and cortical tissue with Trizol reagent following the manufacturer’s instructions (Invitrogen Inc., Carlsbad, CA). Ribonucleic acid in each sample was quantified with the *Nanodrop 1000 Spectrophotometer (Thermo Scientific Inc, Waltham, MA)*. Complimentary deoxynucleic acid was produced from 2 ug of ribonucleic acid using Superscript II (Invitrogen Inc.) with random hexamer primers (Roche Diagnostics Inc., Indianapolis, IN). Amplification was detected with SYBR green using an Applied Biosystems 7900HT Sequence Detection System. Primers against 18S ribonucleic acid (Applied Biosystems Inc, Foster City, CA) were used as an endogenous control for ΔCT calculations. Primer sets were generated against rat carbonic anhydrase-9; erythropoietin; erythropoietin receptor; glucose transporter-1; hypoxia inducible factor-1 alpha; hypoxia inducible factor-2 alpha; nitric oxide synthases (e-endothelial, i-inducible, and n-neuronal); phosphoglycerate kinase-1; and vascular endothelial growth factor. (sequences available on request).

*Western Immunoblotting*

Three to four animals in each group again underwent either sham or true isovolemic hemodilution. Animals were sacrificed at 24 or 48 hours and cortical and hippocampal tissue obtained as described above. Samples from the all animals in each groups were pooled prior to immunoblot analysis due to limited tissue sample volume and desire to test the expression of multiple proteins.

The pooled hippocampal and cortical tissues were homogenized (Polytron, Kinemetica Inc, Bohemia, NY) and sonicated with an ultrasonic dismembranator (Fisher Scientific Inc, Pittsburgh, PA) in Tissue Lysis Reagent (Sigma Inc, St. Louis, MO) with protease inhibitor. Protein concentrations were measured using the Nanodrop Spectrophotometer (Thermo Scientific Inc.). Equal aliquots for each group were separated by reducing [sodium dodecyl sulfate](http://en.wikipedia.org/wiki/Sodium_dodecyl_sulfate) [polyacrylamide gel](http://en.wikipedia.org/wiki/Polyacrylamide_gel) [electrophoresis](http://en.wikipedia.org/wiki/Electrophoresis), transferred to nitrocellulose membranes, blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween20, and immunoblotted with antibodies as follows: Hypoxia inducible factor-1α (1:800, R & D Systems, Minneapolis, MN), vascular endothelial growth factor (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), glucose transporter-1 (1: 200, Santa Cruz Biotechnology, Inc.), and neuronal nitric oxide synthase (1:200, Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-conjugated secondary antibody was used at 1: 5000 (Santa Cruz Biotechnology, Inc.), and signals were detected by SuperSignal West Pico chemiluminescence (Pierce Chemical, Rockford, IL). Plates were scanned and densitometry performed using

ImageJ (Research Services Branch, National Institute of Health, Rockville, MD). Results are presented relative to β-actin or Ponceau loading controls.

***Sample Size Justification***

For Memory Testing, an n = 12-15 animals/group had been estimated as required to test for a hypothesized difference of 15-20% in any performance measure between groups, SD=15%, p=.05, and power=.80. Due to the higher expected mortality in the aged animals during the course of the study, we began the study with 13 animals in the YSh and YAn and 15 animals in the ASh and AAn groups. After study entry, a single animal from the YSh and ASh groups died during recovery from surgery. The total number of rats utilized in this experiment was 54. Final study group numbers then were as follows: 1) YSh, n = 12; 2) YAn, n = 13; 3) ASh, n = 14; and 4) AAn group, n = 15.

For Cerebral Oxygenation, sample size requirements were based on obtaining 80% power, at alpha=0.05, to detect an overall effect size (ratio of hypothesized difference in change in PBrO2 between groups to the standard deviation of the measurement) of 1.0 between the two age groups under an ANOVA model at a given time. Final sample sizes were as follows: YAn, n=14, and AAn, n=16. Testing was terminated in three young SHR and in two aged SHR due to technical complications related to probe or cannula placement.

For Molecular Markers of Hypoxia (Quantitative Real-Time Polymerase Chain Reaction), sample size calculations were based on having sufficient power to detect differences in hypoxic responses by time, age group and hemodilution treatment; in a 2x3x2 design. This study was designed to detect an effect size (=difference in means / standard deviation) of 1.0 when undertaking pair-wise comparisons of group means for main effects, of 1.5 for pair-wise comparisons between two-way interaction effects, and a more modest effect size of 2.0 for pair-wise comparisons between the three-way interaction effects. In order to have 80% power to detect these levels, with alpha=0.05 under a three-way ANOVA model with Tukey adjustments for multiple comparisons, we would require n=10 animals in each of the 12 possible group combinations. One animal in the AAn 24 hour group died prior to brain recovery and analysis. One animal in each of the YSh and MSh 48 hour groups also died prematurely.

## **Statistical Analysis**

For Memory Testing, group means with standard deviations are presented for the multiple measures. Repeated measures ANOVA was used to test for differences in various measures within subgroups over specific time frames and for these analyses exact p-values are presented. For multiple comparisons, statistical significance was tested using Tukey-Kramer HSD (Tukey-HSD) methodology for multiple comparisons with a threshold of p<0.05. For the analysis of the dependence of TTC upon subgroup, a log transformation of the data, ln (TTC) was first carried out to ensure the normality assumption of the statistical tests. For the critical analysis of the role of Group (Aged vs..Young) and treatment SubGroup (Anemia vs. Sham), a linear mixed effects model was implemented to account for the main effects(Group and SubGroup) and to account for the random effects of platform location and unusual influence of any individual animal. A natural log transformation of the TTC data, ln (TTC), was performed as is required when there are unequal variances between the groups prior to the analysis and to result in normally distributed data. The variables animal and platform location were also included in the analysis as random effects to assess whether any particular animal or platform location was particularly influential in the analysis. Only platforms P2-P8 were included in this analysis, as at least 80% of animals in every subgroup successfully attained criteria on all of these platform locations, while only 27% and 0% in the AAn group were ever progressed to allow testing on P9 and P10 respectively.

P-1 was not novel, as it was used for pre and post-hemodilution reference memory testing. Tukey-HSD analysis for multiple comparisons was used to test for differences in TTC between the four study groups. Again, ln (TTC) was used in place of TTC.

For Cerebral Oxygenation, within group means were compared via repeated measures analysis of variance (ANOVA). Comparison of between group means was conducted using the Student’s t-test. Significance was assigned at p ≤ 0.05.

For Molecular Markers of Hypoxia, within group means were tested with two tailed t-test. ANOVA regression analysis was used to test the dependence of each gene level upon 1) An vs. Sh treatment, 2) Age Group, 3) the interaction between the treatment and the Age Group(An x Age Grp), 4) the sampling time (24 vs. 48 hours), and 5) the site of sampling (hippocampus vs. cortex).

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