SUPPLEMENTAL MATERIAL

Rapamycin Induces an eNOS-dependent Increase in Brain Collateral Perfusion in Wistar and Spontaneously Hypertensive Rats

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Supplementary Methods

Animals

Animal experiments were performed on either male outbred Wistar rats or male Spontaneously Hypertensive Rats (SHRs) weighing 300-350 g (Harlan, Bicester, UK). All animals were housed in a 12 h light/dark cycle and had ad libitum access to food and water prior to experiments. All procedures conformed to the Animal (Scientific Procedures) Act 1986 (UK) and the National Institutes of Health guidelines for care and use of laboratory animals and were approved by the University of Oxford Animal Ethics Committee, the Home Office (UK) and the Institutional Animal Care and Use Committee at the University of Vermont. The studies were conducted and the manuscript prepared in accordance with the ARRIVE guidelines.[17]

Anesthesia and Monitoring

The anesthetic and monitoring protocols were as previously reported.[29] Rats were anesthetized with 5% isoflurane in O_2/N_2 (1:3) and maintained with 1% to 2% isoflurane. Core temperature was maintained at 37˚C by a thermocouple rectal probe and warming plate (Harvard Apparatus, UK). Incision sites were shaved, cleaned, and injected subcutaneously with 2mg/kg 0.05% Bupivacaine (Aspen, UK). In SHR studies blood gases were monitored intermittently throughout the experimental procedure. Blood samples (0.1mL) were taken from a right femoral arterial line. This line was also used for continuous arterial pressure monitoring. After stroke surgery, all animals in Wistar experiments were injected subcutaneously with saline (2 x 2.5 ml) to prevent dehydration and returned to their cages with free access to soft sweetened food (Ready Brek with jam and peanut butter) and water. SHRs were euthanized and brains taken for infarct volume determination.

Experimental Stroke Model

Rats underwent MCAo using the silicone-tipped intraluminal thread occlusion method, using 4-0 monofilament occluding threads with 4 mm length x 0.35 mm diameter silicone tips, as previously reported.[18,19] The filament was advanced into the right external carotid artery stump and up the internal carotid artery to occlude the origin of the right MCA. The filament was retracted after 90-120 min to produce recanalization.

Multisite Laser Doppler Flowmetry (LDF)

The animal's head was secured with ear bars in a stereotaxic frame. Probe 1 was placed + 4mm lateral of midline and -2mm posterior of Bregma to measure changes in core MCA CBF . Probe 2 was placed +3mm lateral of midline and +2mm anterior of Bregma to measure changes in CBF within the border zone between the anterior cerebral artery (ACA) and MCA perfusion territories. Previous cerebrovascular casting studies have shown that LMA are consistently located ~2 mm lateral to midline (approximately 1mm upstream of probe 2) in both Wistar and SHRs.[21,22] Furthermore, these coordinates have been validated to measure to measure MCA core and peri-infarct CBF during MCAo using magnetic resonance imaging (MRI)[23] and hydrogen clearance. [24]

Drug Administration

Prior to MCAo, the saphenous branch of the femoral vein was cannulated with 1-French silicon tubing. Rapamycin (Sigma Aldrich, 250μg/kg) or vehicle (<5% ethanol diluted in normal saline) was given as a 1ml bolus after 30 min of ischemia. Treatment with vehicle or rapamycin was randomly assigned by sealed coded numbered envelope. The surgeon was blind to treatment and was blinded during all subsequent analysis of results.

Adhesive Removal Test

The adhesive removal test was used to measure somatosensory deficits following stroke.[27] An adhesive strip (1cm x 1cm) was placed on to the rat's left or right forepaw; care was taken in each trial to apply each stimulus with equal pressure and to randomize the order of application. The time for the animal to touch the adhesive with its mouth (time-to-touch) was recorded. Each animal was given up to 180 s to touch the adhesive. The average of three trials on each paw was taken. Animals underwent training once a day for 3 days prior to stroke, allowing them to acclimatise to the testing environment, reducing stress and optimising their performance on the test. Animals underwent testing immediately prior to MCAo and again at 24 h after MCAo. It is expected that MCA stroke will produce a contralateral forepaw somatosensory deficit reflected by an increase in time-to-touch.[27]

Neurological Deficit Testing

The forelimb flexion, torso twist, and lateral push tests were used to assess this and a total neurologic deficit score given out of 6 (higher score indicating greater deficit).[28]

Histological Analysis of Brain Damage

At 24 h following MCA occlusion, Wistar rats were euthanized and perfused transcardially with saline and then 4% paraformaldehyde. The brains were removed and post-fixed in 4% before being processed, cut into 10 micron coronal sections and stained with haemotoxylin–eosin. Sections were imaged at X 40 using an Aperio slide scanner (Aperio Technologies Inc., Vista, CA, USA) and three regions of interest (ROIs) were traced using Aperio's imagescope software: the stroke hemisphere (ipsilateral to MCA occlusion), the non-stroke hemisphere (contralateral hemisphere ROI) and the infarct area (infarct ROI). Tracing the infarct area under high-power magnification, instead of low power, prevented the under- or over-estimation of the infarct area. Microscopically infarct areas were represented by complete neuronal eosinophilia, with condensed nuclei, cytoplasmic scalloping and no visible nucleolus. Areas of selective neuronal necroses were not included in the infarct ROI. Macroscopically, the infarct area was represented by the area of pallor. The infarct volumes for each animal were calculated by averaging the infarct area between adjacent slices and multiplying by the distance between slices. Volumes were then corrected for edema using the formula: corrected infarct volume (mm^3) = infarct volume x (contralateral volume/ipsilateral volume).

At 4 h following MCA occlusion, SHRs were euthanised and the brain was quickly removed for measurement of infarct volume using 2,3,5-triphenyltetrazolium chloride (TTC) staining. brains were removed and sliced to 2mm coronal sections. Brain sections were incubated for 30 min at 37 ^{o}C in 2% TTC in phosphate buffered saline to stain for infarction, and subsequently fixed in 10% PBS-buffered formalin at 4^{o}C for 45 min before brains were placed on grid paper

(5mm x 5mm) and photographs of both sides of each section were taken. Infarct was identified as a white area of each section and measured with ImageJ software (National Institutes of Health, Bethesda, MD). The grid was used to adjust the scale for each image in ImageJ prior to determination of infarct, ipsilateral hemisphere and contralateral hemisphere areas. The infarct volumes for each animal were calculated by averaging the infarct area on each side of all brain slices and multiplying by the slice thickness and adding the infarct volume of all slices together. Volumes were then corrected for oedema using the formula: corrected infarct volume (mm^3) = infarct volume x (contralateral volume/ ipsilateral volume).[30]

Reactivity of Isolated LMAs

LMAs were identified as distal branches between ACAs and MCAs. They were isolated from male Wistar rats (n = 4) and pressurized in an arteriography bath (Living System Instrumentation, St. Albans, VT), containing circulating physiological saline solution (PSS), as previously described.[16,31] LMAs were equilibrated for 1 hour at 40 mmHg, the time required to develop spontaneously myogenic tone. Then intravascular pressure was increased stepwise in 20 mmHg increments to 80 mmHg to assess myogenic reactivity. Lumen diameter was recorded once stable. Dilation of LMAs to increasing concentrations of rapamycin (10^{-9} to 10^{-5} mol/L, Sigma St Louis, MO) was assessed in the absence and presence of a single concentration of the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 10^{-3} mol/L). Passive structural measurements were obtained in fully relaxed conditions at the end of experiments in zero calcium PSS with added diltiazem (10^{-5} mol/L; MP Biomedicals, Santa Ana, CA) and papaverine (10^{-4} mol/L; Sigma, St. Louis, MO). Passive lumen diameter was used to calculate % tone and % reactivity to rapamycin. Myogenic tone was calculated by the formula: $[1-(\phi_{active}/\phi_{passive})] \times 100\%$; where ϕ_{active} is the lumen diameter of the vessel under active conditions and $\phi_{passive}$ is the lumen diameter of fully relaxed vessel under passive conditions. Percent reactivity was calculated using the formula: $[(\phi_{dose}-\phi_{baseline})/(\phi_{passive}-\phi_{baseline})] \times 100\%$; where ϕ_{dose} is the lumen diameter at a specific concentration of rapamycin.

Detailed Statistics and Sample Size Calculations

Unpaired student's t-test was used to compare differences between the two treatment groups. Differences in LMA or reperfusion CBF between vehicle and rapamycin treatment groups over time were assessed using two-way repeated measures ANOVA. Post-hoc Sidak's and Dunnett's multiple comparisons were used to compare between treatment groups at each time-point and to compare between time-point within treatment groups, respectively. Correlation of LMA and reperfusion CBF with final infarct volumes was analyzed using linear regression. The primary outcome for this study was LMA perfusion. Secondary outcome measures were reperfusion CBF, infarct volume and neurobehavioral scores.

A formal sample size was not determined prior to LMA perfusion measurements with rapamycin treatment in Wistar rats due to unknown outcomes. Given that SHR LMA have greater tone than Wistar LMA under physiological intravascular pressure,[16] we anticipated that they would have at least equivalent or greater potential to dilate in response to rapamycin. Therefore, we planned the SHR study with n= 6/group, based on the maximum effect size (difference between rapamycin and vehicle) and variability of the change in LMA perfusion in

Wistar rats (58% with a standard deviation of 0.35). We were able to reject the null hypothesis that rapamycin does not enhance collateral flow with probability (power) 0.80. The type I error probability associated with the test of this null hypothesis (alpha) was 0.05.

Supplementary Results

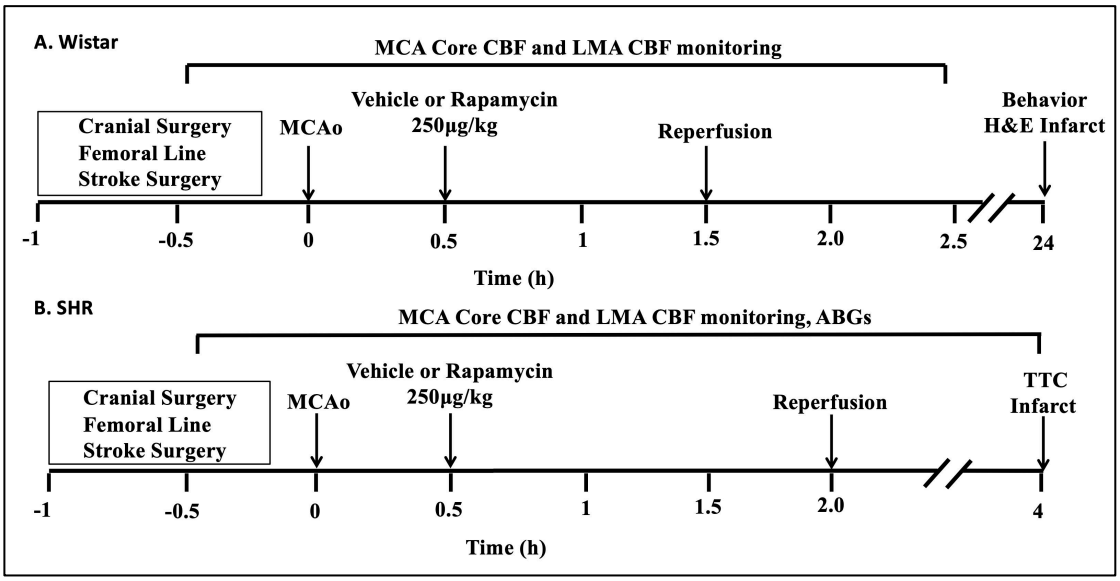
Measurement of distinct perfusion territories with multisite LDF in Wistar Rats

MCAo was confirmed by a 70% reduction in MCA perfusion in LDF probe 1 (core/MCA). We confirmed that we were measuring distinct perfusion territories by comparing changes in CBF between probe 1 and probe 2 (peri-infarct/LMA). Figure 1B shows that the peri-infarct flow (probe 2) was significantly less than core flow (probe 1) (vehicle probe 1: -73.9 ± 1.9 vs. vehicle probe 2: -36 ± 15.2 % of pre-stroke baseline, $p < 0.05$; rapamycin probe 1: -75.9 ± 1.4 vs. rapamycin probe 2: -16.2 ± 11.1 % of pre-stroke baseline, $p < 0.001$), demonstrating that the two probes were measuring different hemodynamic regions.

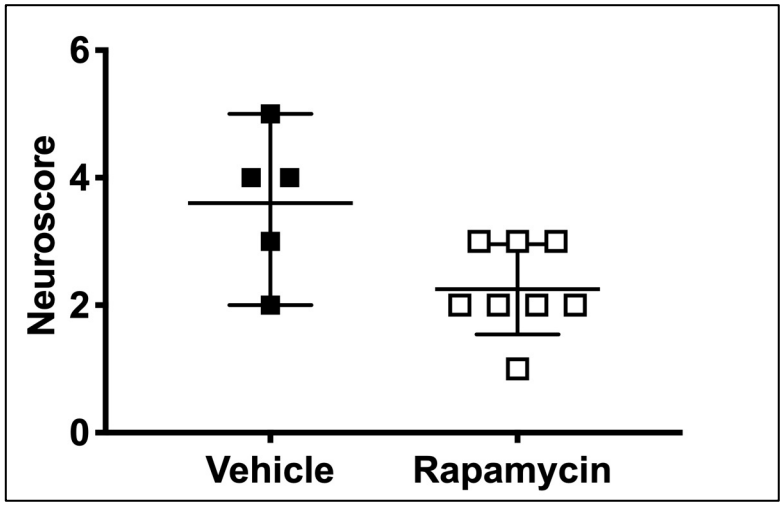
Measurement of distinct perfusion territories with multisite LDF in SHR

MCAo was confirmed by a 70% reduction in MCA perfusion in LDF probe 1 (core/MCA). We confirmed that we were measuring distinct perfusion territories by comparing changes in CBF between probe 1 (MCA) and probe 2 (peri-infarct/LMA). Figure 1C shows that the peri-infarct flow (probe 2) was significantly less than core flow (probe 1) (vehicle probe 1: -80.1 ± 2.8 vs. vehicle probe 2: -30.4 ± 9.1 % of pre-stroke baseline, $p < 0.001$; rapamycin probe 1: -81.5 ± 3.9 vs. rapamycin probe 2: -39.6 ± 9.7 % of pre-stroke baseline, $p < 0.01$), demonstrating that the two probes were measuring different hemodynamic regions.

Supplementary Figures



Supplementary Figure I. Experimental timelines. *(A) Wistars. MCA and LMA CBF were monitored before and after administration of 250µg/kg of rapamycin at 30 min post-MCAo in Wistar rats. (B) MCA and LMA perfusion were monitored before and after administration of 250µg/kg of rapamycin at 30 min post MCAo onset in SHRs. ABG, arterial blood gas; CBF, cerebral blood flow; LMAs, leptomeningeal anastomoses; MAP. Mean arterial pressure, MCAo, middle cerebral artery occlusion.*



Supplementary Figure II. Neurological deficits after stroke in Wistar rats.

*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

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:	Yes
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:	Yes
An overall study timeline is provided:	Yes

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

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:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	Yes

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:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

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:	Yes

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:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
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:	Yes

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:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	No

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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