Calcium-dependent blood-brain barrier breakdown by NOX5 limits post-reperfusion benefit in stroke

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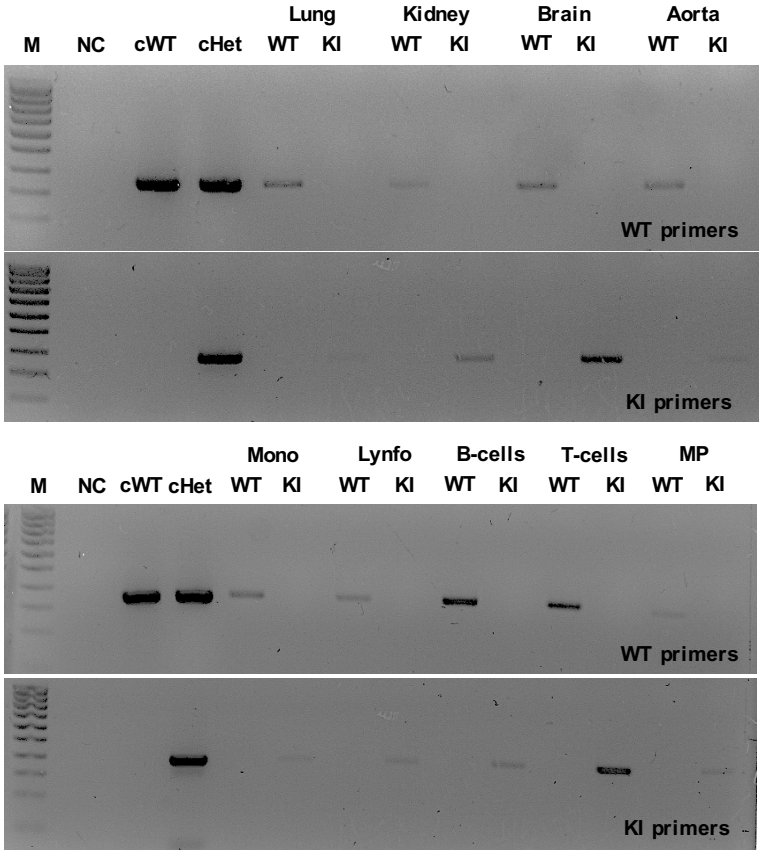
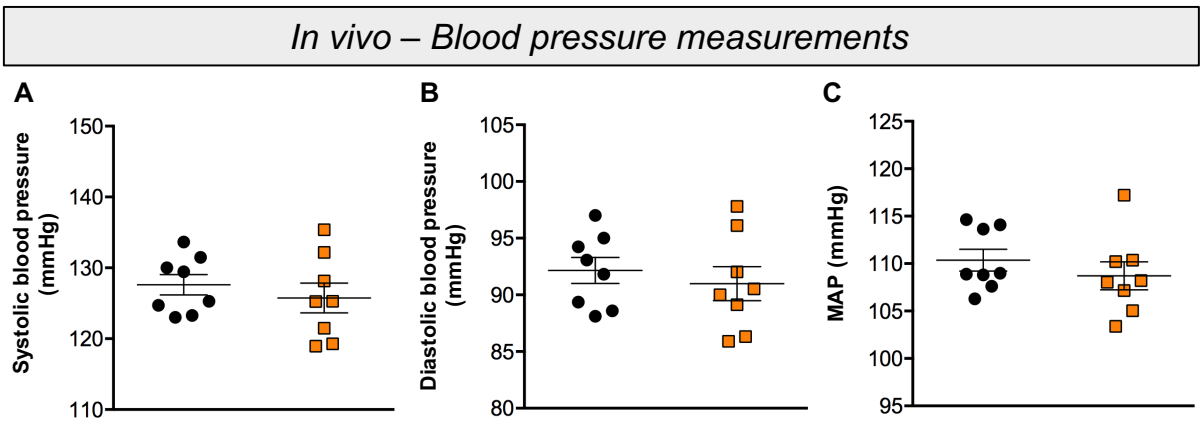
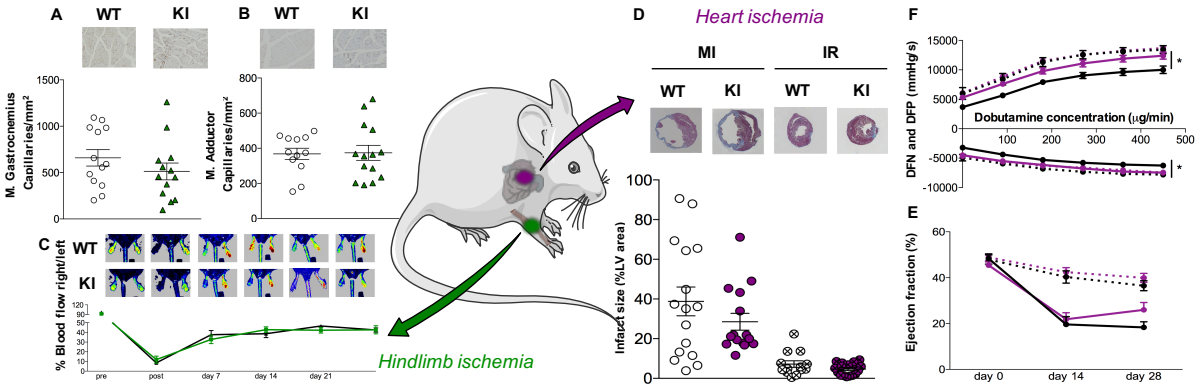
Supplementary Figures



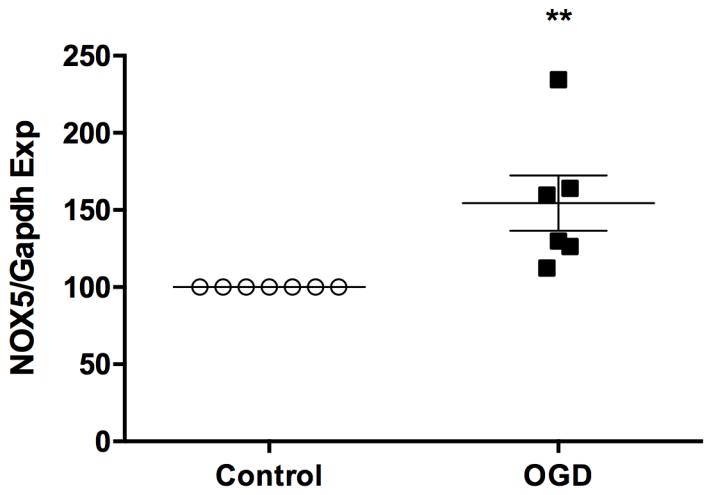
Supplemental Figure 1. NOX5KI mice genotyping. Control (WT) and NOX5KI mice tail genomic DNA were purified and a PCR was performed to amplify the *NOX5* sequence in different tissues such as lung, kidney, brain, aorta and white blood cells (monocytes, lymphocytes, B- and T-cells). *NOX5* DNA sequence were detected in NOX5KI mice while no signal was shown in WT mice.



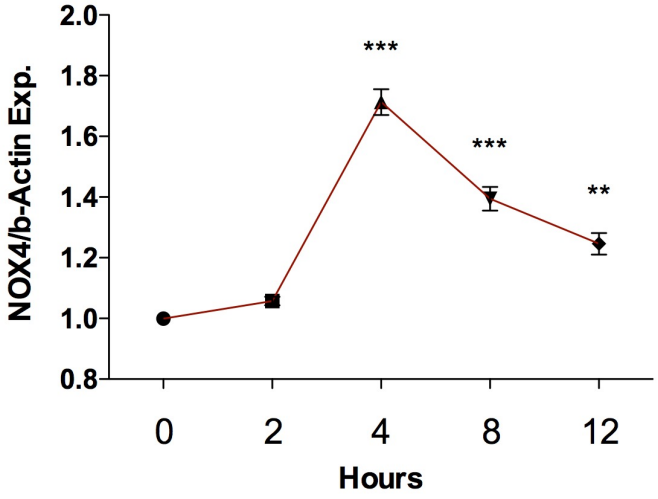
Supplemental Figure 2. No role of NOX5 in blood pressure. (**A**) Systolic blood pressure was not different between WT (black, n = 19) and NOX5KI mice (orange, n = 20). (**B**) Diastolic blood pressure and (**C**) mean arterial pressure (MAP) did not differ between WT (black, n = 19) and NOX5KI (orange, n = 20) mice during day-time (07:00-18:00) or night-time (18:00-06:00).



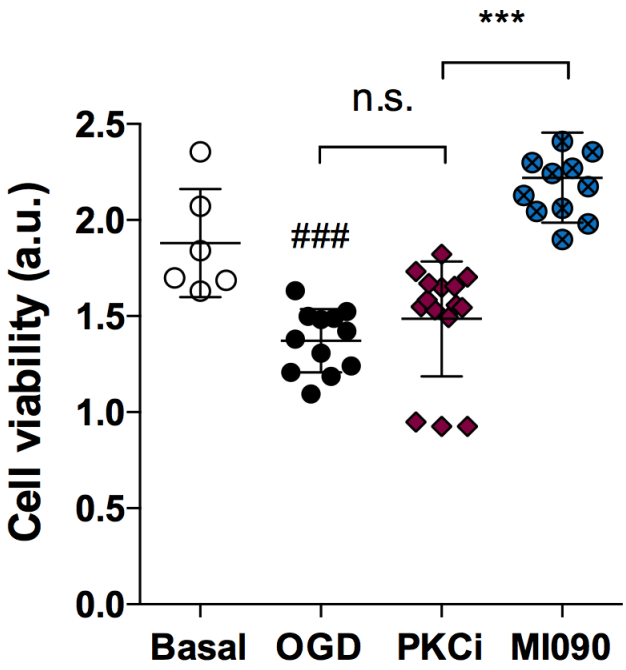
Supplemental Figure 3. No role of NOX5 in three different cardiovascular ischemic models. Capillary density (capillaries/mm^3, 40X magnification) as measured by CD31 staining showed no difference between NOX5KI (green) and WT (black) mice 4-weeks after ligation of the femoral artery in (**A**) gastrocnemius (KI, n=13; WT, n=13) (**B**) and adductor muscles (KI, n=14; WT, n=12). (**C**) Blood flow restoration after ligation of the left femoral artery was not different between WT (black, n = 13) and NOX5KI (green, n=10) mice. (**D**) Infarct size (10X magnification) was not different between WT (black, n=16) and NOX5KI mice (purple, n=15) in the myocardial infarction model. Similarly, no difference was shown in the transient ischemia-reperfusion heart model (hatched black, n=13; hatched purple, n=17). (**E**) Ejection fraction decreased 2- and 4-weeks after permanent (full lines) or transient (dashed lines) heart ischemia. No differences were found between WT (black, n=16; hatched, n=12) and NOX5KI mice (purple, n=14; hatched, n=16). (**F**) Functional measurements of the heart showed better contractile and relaxing properties of the NOX5KI hearts 4-weeks after permanent ligation (full purple lines, n=15) compared to WT (full black lines, n=13) (* $P < 0.05$, 2-tailed unpaired t test). In transient ischemia (dashed lines; WT, n=12; KI, n=17), no difference was seen. Data represent as mean ± SEM in all experiments.



Supplemental Figure 4. *NOX5* expression in human brain microvascular endothelial cells (HBMECs) under ischemic conditions. *NOX5* gene expression in HBMECs subjected to 6h of hypoxia (0.2% O_2) was significantly increased in comparison with HBMEC subjected to normoxia conditions ($^{*}P<0.05$, n=5, 2-tailed unpaired t test).



Supplemental Figure 5. NADPH oxidases 4 (NOX4) are up-regulated at different time-points after oxygen and glucose deprivation (OGD). Organotypic hippocampal cultures (OHCs) prepared from mice hippocampal brain slices were cultured for 4 days and subsequently subjected to 15 min of OGD period. Brain slices were collected at 0, 2, 4, 8, and 12 after OGD for later gene expression analysis. *NOX4* expression was up-regulated at 4h, 8h and 12h in comparison with the beginning of the ischemia period ($^{**}p < 0.01$, $^{***}p < 0.001$, n = 3, 2-tailed unpaired t test).



Supplemental Figure 6. PKC inhibition before re-oxygenation show no effect in cell viability using a human in vitro ischemia model. Cell viability was assessed by measuring MTT fluorescence 24h post-hypoxia. Cell viability was significantly increased in cells subjected to early treatment of MI090 (0.01μM), (*** $P < 0.001$, 2-tailed unpaired t test) compared to non-treated cells ($^{###} P < 0.001$). No significant effect was detected while inhibiting PKC (GF 109203X, 0.3 μM).

Supplementary tables

Table S1. Frequencies (mean value) of leukocyte subpopulations in peripheral blood of NOX5KI mice vs WT under baseline conditions

	Female		Male		Lineal model
Flowcytometric measurements	*Control* n=13 mean ± sd	*Mutant* n=15 mean ± sd	*Control* n=9 mean ± sd	*Mutant* n=19 mean ± sd	*Genotype* p-value
$CD45^+$/T cells	26.6 ± 3.85	27.07 ± 5.26	24.36 ± 3.51	23.27 ± 7.31	0.85
$CD45^+/CD3^+CD4^+$	12.73 ± 2.07	14.14 ± 2.92	12.96 ± 2.32	11.51 ± 3.71	0.984
$CD45^+/CD3^+CD8^+$	9.62 ± 1.87	8.5. ± 1.95	8.52 ± 1.47	7.79 ± 1.71	0.158
$CD45^+$/B cells	33.39 ± 6.74	32.82 ± 4.48	34.69 ± 4.28	31.14 ± 5.43	0.2
$CD45^+$/CD5-NK^+	3.85 ± 1.12	4.33 ± 1.22	7.29 ± 10.24	4.44 ± 1.41	0.288
$CD45^+/CD11b^+Grl^+$	11.01 ± 3.66	10.57 ± 3.53	12.97 ± 10.27	10.96± 4.53	0.424
$CD45^+$/NK-Grl-$CD11b^+$	14.76 ± 5.58	14.39 ± 4.23	12.51 ± 3.97	16.54 ± 4.87	0.196
$CD3^+CD4^+/CD25^+$	8.17 ± 2.56	9.32 ± 3.28	7.24 ± 1.97	9.89 ± 8.81	0.264
$CD45^+/CD3^+$gammadeltaTCR$^+$	1.97 ± 1.14	1.49 ± 0.69	1.12 ± 0.49	1.42 ± 1.88	0.812
$CD3^+CD4^+/CD62L^+$	72.12 ±7.41	74.69 ± 8.78	54.67 ± 12.4	55.54 ± 11.72	0.562
$CD3^+CD4^+/CD44^+$	2.82 ± 1.29	2.43 ± 1.07	1.72 ± 1	4.98 ± 12.69	0.516
$CD3^+CD8^+/CD62L^+$	83.28 ± 5.27	85.37 ± 7.52	72.39 ± 9.25	74.63 ± 8.3	0.331
$CD3^+CD8^+/CD44^+$	6.11 ± 2.73	5.62 ± 2.49	4.24 ± 2.06	4.76 ± 3.13	0.987
$CD45^+/CD5^+NK^+$	1.98 ± 1.16	2 ± 1.29	1.68 ± 0.98	1.8 ± 1.05	0.84
Bcells/IgD^+	83.46 ± 3.66	82.67 ± 4.44	89.11 ± 4.13	86.97 ± 2.65	0.178
Bcells/$B220^+MHCclassII^+$	93.69 ± 1.32	93.03 ± 2.44	94.49 ± 1.57	94.17 ± 1.87	0.388
$NK^+/CD11b^+$	94.62 ± 1.67	95.29 ± 2.09	93.86 ± 3.83	93.58 ± 2.17	0.771
$CD3^+$ rest	4.25 ± 0.65	4.07 ± 1.11	2.88 ± 0.51	3.97 ± 3.3	0.452
$CD45^+/CD3^+CD4^+CD25^+$	1.02 ± 0.29	1.32 ± 0.58	0.91 ± 0.13	1.34 ± 2.18	0.362
Ratio Granulocites:(Tcells+Bcells)	0.19 ± 0.08	0.18 ± 0.07	0.16 ± 0.02	0.2 ± 0.08	0.39
Ratio CD4:CD8	1.35 ± 0.21	1.62 ± 0.3	1.53 ± 0.14	1.49 ± 0.33	0.145
Ratio Tcells:Bcells	0.83 ± 0.21	0.85 ± 0.26	0.74 ± 0.21	0.78 ± 0.31	0.665

Table S2. Frequencies (median value) of main leukocyte subpopulations in peripheral blood of NOX5KI mice vs WT under baseline conditions

	Female		Male		Female	Male	Overall
Flowcytometric measurements	*Control* n=13 median	*Mutant* n=15 median	*Control* n=9 median	*Mutant* n=19 median	p-value	p-value	p-value
$CD45^+$/T cells	25.7	26.1	24.6	21.85	0.865	0.303	0.309
$CD45^+/CD3^+CD4^+$	12.1	13.3	12.6	10.55	0.074	0.093	0.686
$CD45^+/CD3^+CD8^+$	9.53	9.1	8.32	7.87	0.406	0.239	0.087
$CD45^+$/B cells	30.5	33.4	36.3	30.65	0.777	0.216	0.332
$CD45^+$/CD5-NK^+	3.99	3.95	3.85	4.31	0.496	0.458	0.212
$CD45^+/CD11b^+Grl^+$	10.6	10.2	8.93	9.28	1	0.79	0.953
$CD45^+$/NK-Grl-$CD11b^+$	14.6	13.1	13	14.2	1	0.128	0.318
$CD3^+CD4^+/CD25^+$	8.45	8.58	6.86	7.62	0.581	0.364	0.333
$CD45^+/CD3^+$gammadeltaTCR$^+$	1.83	1.5	0.98	0.92	0.3	0.824	0.118
Ratio CD4:CD8	1.33	1.6	1.49	1.5	0.011	0.487	0.142
Ratio Granulocites:(Tcells+Bcells)	0.18	0.17	0.16	0.18	1	0.193	0.397
Ratio Tcells:Bcells	0.76	0.78	0.69	0.72	0.901	0.961	0.914

Table S3. Study design. Animals

Model	Measurement	Strain	Drop-out	Outliers	N
Hindlimb ischemia	*Doppler*	WT	0	0	13
		KI	0	3	10
	Capillary density gastrocnemius	WT	0	0	13
		KI	0	1	13
	Capillary density adductor	WT	0	1	12
		KI	0	0	14
Heart ischemia permanent	*Infarct size*	WT	12	0	16
		KI	12	1	15
	DFP	WT	12	1	13
		KI	12	1	15
	DFN	WT	12	1	13
		KI	12	1	15
	Ejection fraction	WT	12	0	16
		KI	12	0	14
Heart ischemia transient	*Infarct size*	WT	1	1	13
		KI	1	0	17
	DFP	WT	1	1	12
		KI	1	1	17
	DFN	WT	1	1	12
		KI	1	2	17
	Ejection fraction	WT	1	1	12
		KI	0	0	16
Blood pressure	*All measurements*	WT	2	0	19
		KI	0	0	20
Stroke	*Infarct size*	WT	5	1	27
		KI	5	4	25
	Blood-brain barrier	WT	0	1	22
		KI	3	2	26

WT, wild type; KI, knock-in.

Table S4. Study design. Power analysis

Infarct size (tMCAO) – NOX5 KI vs WT mice

	Mean	*SD*	*N*	*Power for measured difference (%)*
WT	59,7	20,4	21	99,9%
NOX5 KI	91,6	24	17	

tMCAO, transient middle cerebral artery occlusion; SD, standard deviation; N, number of animals. We conducted a post hoc analysis of power in the different animal groups. For each animal group, a pooled variance of the vehicle and treatment groups was calculated from mean, SD and n-number with n the size of the group and CV the coefficient of variation (SD/Mean) of the group. Power was calculated for the measured difference using Russ Lenth's power software with an alpha of 0.05, the measured effect (%) and the calculated pooled variances [(Lenth, R.V 2006-9, java Applets for Power and Sample Size [Computer Software], Retrieved 02-17-2014.

Supplemental Methods

Generation of the NOX5 knock-in (KI) mouse. Since the rodent genome naturally lacks the *NADPH oxidase 5* gene, we created a new mouse line expressing the human *NOX5* gene under the control of the *Tie2* promoter. The model was developed using the *hypoxanthine phospho-ribosyl-transferase* (*Hprt*) targeted transgenic approach. This locus protects transgenic constructs inserted into this region against gene silencing, positional or methylation effects, and tissue-specific promoters inserted in the *Hprt* locus conserve their expression patterns. Importantly, *Hprt*-deficient mice are phenotypically normal. Targeted insertion at the *Hprt* locus overcomes the unpredictable position effects inherent in transgenic methods relying on random integration. Thus, a respective KI line allows experiments to be conducted with a single mouse line, in contrast to the 3-5 transgenic lines usually required using classical technology. The Hprt gene is localised on the X chromosome, necessitating analysis of hemizygous males or homozygous females. The targeting vector was obtained by subcloning of a transgenic targeting cassette containing the human NOX5 beta gene under control of the Tie2 promoter into the Hprt targeting vector (GenOway, Lyon, France), upstream of the human Growth Hormone polyA sequence. E14 ES cells of 129SV mice were transfected with the linearised targeting vector, selected with HAT (hypoxanthine-aminopterin-thymidine medium) medium and resistant clones screened (Southern blot) for presence of the 3' and 5' homologous recombination events. Selected ES cells were injected into C57Bl6/J blastocysts and re-implanted into OF1 (oestrus phase) pseudo-pregnant females, which resulted in chimeric males. Chimeras were mated with WT C57Bl/6 females and agouti female offspring was genotyped to confirm the presence of the recombined X chromosome. The heterozygous females were then mated with transmitting male chimeras to generate

hemizygous males and homozygous females. All procedures were carried out under SPF conditions by GenOway, which houses all mice at Charles River.

Validation of the NOX5 knock-in (KI) mouse. Isolation of bone marrow macrophages: Bone marrow was isolated and cells were cultured for one week in RPMI-1640 with Glutamac, 2g/L glucose (Gibco, ThermoFisher Scientific, Munich, Germany) supplemented with 10% fetal cow serum (FCS, Sigma-Aldrich, Maastricht, The Netherlands), 100U/mL Penicillin-Streptomycin, and 15% L929-conditioned medium to generate bone-marrow-derived macrophages. Cells were then dissociated with lidocaine (200mg, 1mL 0.5M EDTA in 50mL PBS) before differentiating them with EtOh, IFN-γ and IL-4 to become M0, M1 and M2a subtype macrophages respectively.

Flow cytometric assessment of the frequencies of leukocyte subpopulations in peripheral blood (PBLs): PBLs are characterized from whole blood samples a 10-20µl per mouse. Each whole blood sample is incubated at 4-10°C for 5 minutes with Fc block (clone 2.4G8.14; Elisabeth Kremmer, Helmholtz-Zentrum München). Then, an antibody mix is added and incubated at 4-10°C for 15-60 minutes (antibodies from Becton Dickinson, USA) and an erythrocyte-lysis and a formalin-fixation (BD FACS Lysing solution, Becton Dickinson, USA) is performed. Finally, after washing with FACS staining buffer (PBS, 0.5 % BSA, 0.02 % sodium azide, pH 7.45) samples are acquired from a 96 well plate using a HyperCyt sampler (IntelliCyt Corp., USA) and a 10-color flow cytometer (Gallios, Beckman Coulter, USA). The acquisition threshold is set on the CD45-channel. Our analyses are based on approximately 10.000- 50.000 leukocytes per sample. Frequencies of leukocyte populations are determined by software based analysis (Flowjo, TreeStar Inc, USA). The frequencies of main leukocyte subsets are referred to the CD45+ gate (most CD45 negative cells are

already excluded via the CD45 threshold). Gates for each parameter are based on formerly performed 'fluorescence minus one' (FMO) controls.

Microvascular brain endothelial cell isolation: Brain capillary endothelial cells (MBCEC) from eNOX4 KO and WT mice were isolated as described in (26, 27). After sacrificing the mice, forebrains were collected, meninges removed and the tissue was minced and digested with a mixture of 0.75mg/ml collagenase CLS2 (Worthington, USA) and 10U/mL DNAse (Sigma-Aldrich, Munich, Germany) in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, Munich, Germany) for 1h at 37°C. To remove myelin, the pellet was re-suspended in BSA-DMEM (20% w/v) and centrifuged (1000x g, 20 min). The pellet was re-suspended and further digested with 1mg/ml collagenase-dispase (Roche, Munich, Germany) and 10U/ml SNAse in DMED for 1h at 37°C. Microvascular endothelial capillaries were separated on a 33% continuous Percoll gradient, collected and plated in petri-dishes coated with collagen IV/fibronectin (Sigma-Aldrich, Munich, Germany). Cultures were maintained in DMEM supplemented with 20% plasma-derived bovine serum (First Link, Munich, Germany), 50µg/ml gentamicin (Sigma-Aldrich, Munich, Germany), 2mM L-glutamine (Sigma-Aldrich, Munich, Germany), 4µg/ml puromycin (Alexix GmbH, Grünberg, Germany) and 1ng/ml basic fibroblast growth factor (Roche, Munich, Germany).

Immunology screen: Leukocyte count. Using a 10 color polychromatic flow cytometry assay cells were first discriminated in alive or dead cells followed by identification of T cells, B cells, granulocytes, NK cells, and further subsets within a $CD45^+$ gate.

Organotypic hippocampal culture (OHCs) of NOX5 KI mice. Hippocampal brain slices for cultures were obtained from brains of 7- to 10-day-old WT and NOX5 KI mice. Organotypic cultures were prepared based on the methods previously described in (28, 29). Briefly, pups were quickly decapitated and brains removed from the skull and

dissected. The hippocampus was cut into 300μm-thick slices using a Tissue Chopper McIlwain (WPI, Madrid, Spain). Then, they were separated in sterile ice-cold Hank's balanced salt solution (HBSS, Biowest, Madrid, Spain) containing (in mM): glucose 15, $CaCl_2$ 1.3, KCl 5.36, NaCl 137.93, KH_2PO_4 0.44, Na_2HPO_4 0.34, $MgCl_2$ 0.49, $MgSO_4$ 0.44, $NaHCO_3$ 4.1, HEPES 25, 100 U/ml penicillin, and 0.100 mg/ml gentamicin. Six slices were placed on each Millicell-0.4 μm culture inserts (Millipore, Madrid, Spain) within each well of a six-well culture plate. Specific neurobasal medium (Invitrogen, Madrid, Spain) enriched with 10% of fetal bovine serum (Sigma-Aldrich, Madrid, Spain) was used for the next 24h (1 ml/well). 24h later, B27 supplement and antioxidants were added to the culture medium. Slices were in culture for 4 days before inducing the oxygen and glucose deprivation (OGD) period. On day 6, inserts were placed in 1 ml of OGD solution composed of (in mM): NaCl 137.93, KCl 5.36, $CaCl_2$ 2, $MgSO_4$ 1.19, $NaHCO_3$ 26, KH_2PO_4 1.18, and 2-deoxyglucose 11 (Sigma-Aldrich, Madrid, Spain). The cultures were then placed in an airtight chamber (Billups and Rothenberg, San Diego, USA) and exposed during 3 min to 95% N_2/5% CO_2 gas flow to ensure oxygen removal. After that, the chamber was sealed for 15 min at 37°C (OGD period). After 15 minutes, the cultures were returned to normal oxygen and glucose concentrations for 24h (Re-Ox period).

In vitro ROS formation in organotypic hippocampal cultures. ROS production was evaluated in real-time by the fluorescence dye dihidroethidium (30, 31) (Thermo Fisher Scientific, Maastricht, The Netherlands). A stock solution of DHE (3.2 mM) was dissolved in Krebs solution and added to the culture insert. Fluorescence measurements were performed at 0, 15 min, 30 min, 1h, 2h and 4h after the OGD period in both WT and NOX5 KI mice using a 10X objective in the CA1 region of the hippocampus. Same emission and excitation wavelength were used. Fluorescence

analysis was performed using the Metamorph software version 7.0.

Ca^{2+} overload in organotypic hippocampal cultures. Hippocampal brain slices were in culture 4 days as previously described. To induce Ca^{2+} overload, 10 µM of ionophore A23187 (Sigma-Aldrich, Maastricht, The Netherlands) was directly added to the culture medium and ROS formation was subsequently measured objective in the CA1 region of the hippocampus at 0, 15 and 30 min post-A23187 addition. Fluorescence analysis was performed using the Metamorph software version 7.0. Different time-points were selected based on previously described ROS kinetics.

Transient occlusion of the middle cerebral artery (tMCAO model). The model has previously been established as described in (25). Animals were anesthetized with isoflurane (1.5-2% in oxygen). The animal was placed on a heating-pad, and rectal temperature was maintained at 37.0°C using a feedback-controlled infrared lamp. Focal cerebral ischemia was induced using an intraluminal filament technique. Using a surgical microscope (Wild M5A, Wild Heerbrugg, Gais, CH, USA), a midline neck incision was made and the right common and external carotid arteries were isolated and permanently ligated. A microvascular clip was temporarily placed on the internal carotid artery. A silicon rubber-coated 6.0 nylon monofilament (602312PK10, Doccol Corporation, Sharon, MA, USA) for mice was inserted through a small incision into the common carotid artery and advanced into the internal carotid artery until a resistance is felt. The tip of the monofilament should be located intracranially at the origin of the right middle cerebral artery and thereby interrupting blood flow. The filament was held in place by a tourniquet suture that has been prepared before to prevent dislocation during the ischemia period and the wound was closed. Reperfusion was initiated 30 minutes after occlusion. After the surgery, wounds were carefully sutured and animals recovered from surgery in a temperature-controlled environment. Animals were

excluded from the stroke analysis if animals died before the predefined experimental end-point, if an intracerebral hemorrhage occurred or if the animal scored 0 on the Bederson score.

Brain infarct volume measurements. The ischemic lesion was measured 24 hours after tMCAO using TTC staining (32). The brain was cut in three 2 mm thick coronal sections using a mouse brain slice matrix (Harvard Apparatus, Holliston, MA, USA). The slices were soaked for 10 min in a freshly-prepared solution of 2% 2,3,5-triphenyltetrazolium hydrochloride (TTC, Sigma-Aldrich, Würzburg/Maastricht, Germany/The Netherlands). Total indirect (i.e corrected for brain edema) infarct volume was calculated by volumetry (ImageJ 1.49 software, National Institutes of Health) according to the formula: $V_{indirect}$ (mm^3) = $V_{infarct}$ x (1-(V_{ih}-V_{ch}) / V_{ch}, where the term V_{ih}-V_{ch} represents the volume difference between the ipsilateral and contralateral hemisphere and (V_i-V_c) / V_c expresses this difference as % of the control hemisphere.

Neurological behaviour. The mice were assessed for neurological behaviour just before sacrifice to determine the final functional status. Neurological deficits were measured in a blinded manner on a 0 to 5 scale using the Bederson Score (32) with the following definitions: Score 0, no apparent neurological deficits; 1, body torsion and forelimb flexion; 2, right side weakness and thus decreased resistance to lateral push; 3, unidirectional circling behaviour; 4, longitudinal spinning; 5, no movement.

Motor function. Prior to sacrifice, the mice were also scored for neurological motor deficits according to the Grip Test (25). Each mouse was given a discrete value from 0 to 5. This score is used to evaluate motor function and coordination. The apparatus is a metal rod (0.22 cm diameter, 50cm length) between two vertical supports at a height of 40 cm over a flat surface. The animal is placed mid-way on this rod and is rated according to the following system: Score 0, falls off; 1, hangs on to string by one

or both fore paws; 2, as for 1, and attempts to climb on to string; 3, hangs on to string by one or both fore paws plus one or both hind paws; 4, hangs on to string by fore and hind paws plus tail wrapped around string; 5, escape (towards the supports).

Blood-brain barrier function. To determine the permeability of the cerebral vasculature and brain edema, 2% Evans blue tracer (Sigma-Aldrich, Würzburg/Maastricht, Germany/The Netherlands) diluted in 0.9% NaCl was injected i.p. at reperfusion. Measurement of Evans Blue extravasation was performed as described in (25).

Oxidative stress: DHE staining. The presence of ROS was determined using dihydroethidium, stock solution 2 mM (Sigma-Aldrich, Würzburg, Germany) staining in coronal brain sections taken from identical regions (-0.5mm from bregma) of the different animal groups. Briefly, frozen sections were incubated in 2μM DHE for 30 minutes at 37°C, washed three times with PBS and incubated with Hoechst (Hoechst 33342, Sigma-Aldrich, Würzburg, Germany) 2 ng/ml for 10 min. All sections were analyzed and acquired with a Nikon Eclipse 50i microscope equipped with the DS-U3 DS camera control unit. The relative pixel intensity was measured in identical regions with NIS- Elements software (Nikon, Tokyo, Japan). Digital images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Heart ischemia: Myocardial infarction and ischemia reperfusion. Mice aged 8-16 weeks were subjected to permanent or transient ligation of the left descending coronary artery. After administration of an analgesic (buprenorphine s.c. 0.05mg/kg, Temgesic, Schering-Plough, USA), mice were anaesthetized with isoflurane (Abbott forene Isoflurane, Maastricht, The Netherlands) 4-5% in air and intubated per orally with a stainless-steel tube. The tube was connected to a respirator (rodent ventilator Microvent type 845, Hugo Sachs Electronic, Germany), set at a stroke volume of 250μL and a rate of 210 strokes/min. Anaesthesia was then maintained with 2-3%

isoflurane in air via a vaporizer (Univentor, UNO Roestvaststaal BV, Germany) connected to the respirator. The mouse was placed on a heating pad (UNO temperature control unit, UNO Roestvaststaal BV, Germany) and body temperature was monitored using a rectal probe and maintained at 37.0°C using a feedback-controlled infrared light. During surgery, an ECG was recorded with IDEEQ software (IDEE, Maastricht University, The Netherlands). A left thoracotomy was performed to expose the heart. Then, the left descending coronary artery (LAD) was ligated with a 6-0 polypropylene suture (Surgipo, Chicago, IL, USA), just proximal to its main branching point. The suture was tied around a 3 mm-long polyethylene tube (PE-10) to induce ischemia. Ischemia was assessed by a discolouration of the tissue (from red to pink-white) and ST-elevation on ECG. After 45 minutes, the blood flow was re-established by removal of the polyethylene-tube. The occurrence of reperfusion was assessed by the colour of the tissue turning red again. For the myocardial infarct model, the LAD was ligated with a 6-0 polypropylene suture permanently. The chest was closed with 5-0 silk sutures (Ethicon, USA). The animals were weaned from the respirator and the endotracheal tube was removed, once the mice breathed spontaneously. After surgery, mice were allowed to recover at thermoneutral temperature (28°C). In the morning and afternoon of the day after, s.c. 0.05 mg/kg buprenorphine hydrochloride was repeated to relieve pain.

Evaluation of infarct size in heart. After the hemodynamic measurements at day 28, the heart was excised and the atria were removed. The ventricles were cut transversally at 3 mm from the apex. The apical part was fixed in formalin and processed for paraffin embedding. Paraffin sections of 4 μm were stained with AZAN (infarct size) or Picrosirius red (collagen content). Images were visualised under light microscopy (Zeiss Axioskop microscope, Germany) and digitalised using a Leica

DFC490 camera (Leica Microsystems Ltd, Heerbrugg, Germany) Pictures were analysed using the Leica Qwin pro v3.5.1 software. Infarct sizes are expressed as percentage area of the total left ventricular tissue area. Animals with no visible infarct were deleted from all analysis.

Ligation of the femoral artery. Directly after a basal Doppler measurement, anaesthesia was maintained with 1.5-2% isoflurane. The mouse was placed on the back on a heating pad (UNO temperature control unit, UNO Roestvaststaal BV, Zevenaar, The Netherlands) and body temperature was monitored using a rectal probe and maintained at 37.0°C using a feedback-controlled infrared light. The right groin was disinfected and the right femoral artery was ligated by placing a suture (5-0 silk) around the femoral artery in between the branching of the a. epigastrica and the a. poplitea. These last two arteries were also ligated to prevent collateral flow and backflow respectively. The wound was then closed with a 4-0 polysorb suture and the mouse was allowed to recover. Discoloration of the paw was visible after the ligating surgery and an accompanying drop in blood flow was visible with laser Doppler.

Capillary density. After the last Doppler measurement at day 28, the musculus adductor and musculus gastrocnemius were prepared free, dissected and formalin fixed. Paraffin embedded sections of 4 μm were stained for capillary cells, using a monoclonal rat anti-mouse antibody to CD31 (PECAM-1, Histonova-Dianova, Cat. no DIA310, Hamburg, Germany) diluted 1:50. As secondary antibody, biotin labelled rabbit anti-rat antibody (dakocytomotion Denmark no. E0468, Dako, The Netherlands) was used diluted 1:200. Images were visualised under light microscopy (Zeiss Axioskop microscope, The Netherlands) and digitalised using a Leica DFC490 camera (Leica Microsystems Ltd, Heerbrugg, USA). Pictures were analysed using the Leica

Qwin pro v3.5.1 software. For each animal, three random pictures were taken per muscle sample and the amount of capillaries is expressed as number per mm^2.

Human brain microvascular endothelial cells (HBMECs) subjected to hypoxia. Primary culture HBMEC (cat. N. ACBRI_376_V, Cell systems, USA) between passage 3 and 9 were cultured to approximately 95% confluence using recommended cell medium (EGM-2 MV BulletKit, Lonza, Maastricht, The Netherlands) enriched with 5% fetal bovine serum (FBS; Sigma-Aldrich, Maastricht, The Netherlands) before starting the experiment. For hypoxia studies, HBMECs were always seeded at specific cell density ($6x10^4$ cells/ml) in 12 wells-plate and incubated during 24h at 37°C. Then, cell medium was replaced with non-FBS enriched medium (2 ml/well) following by 6h of hypoxia (94,8% N_2, 0.2% O_2 and 5% CO_2) at 37°C using hypoxia workstations (Ruskin Invivo2 400 station, Maastricht, The Netherlands). The hypoxia period was followed by 24h of re-oxygenation at 37°C, enriched medium and normoxia conditions (75% N_2, 20% O_2 and 5% CO_2) in the presence or absence of treatment. All culture surfaces were pre-treated with fibronectin solution (1:100 in PBS, Sigma-Aldrich, Maastricht, The Netherlands).

HBMECs were treated at two different time points: early treatment (25 min before re-oxygenation) and late treatment (25 min after re-oxygenation) with 0.01μM MI090 (NOX5 inhibitor) and 0.2μM M13 (NOX4 inhibitor, Glucox, Sweden).

Assessment of cell permeability in HBMECs. 2 x 10^4 HBMECs were seeded and incubated during 24h on Transwell inserts (collagen-coated Transwell Pore Polyester Membrane Insert; pore size = 3.0 μm, Corning, Maastricht, The Netherlands) before inducing 6h of hypoxia (94,8% N_2, 0.2% O_2 and 5% CO_2) followed by 24h re-oxygenation period in presence/absence of early or late treatment. The Evans Blue dye (Sigma-Aldrich, Madrid, The Netherlands) was used to assess cell permeability

on the insert. Before the diffusion experiment, the medium was removed and cells were washed once with assay buffer (37°C-warm PBS). 1.5 ml of the same buffer was added to the ab-luminal side of the insert. Permeability buffer (0.5 ml) containing 4% bovine serum albumin (Sigma-Aldrich, Maastricht, The Netherlands) and 0.67 mg/ml Evans blue dye in PBS was loaded on the luminal side of the insert followed by 15 min incubation at 37°C. Evans Blue concentration in the ab-luminal chamber was measured by determining the absorbance of 150 µl buffer at 630 nm using a spectrophotometer.

Statistics. All data are expressed as mean ± SEM. Using the GraphPad Prism 6.0 software package data were assessed for normal distribution using the D'Agostino & Person omnibus normality test. For each outcome parameter, outliers clearly >2SD were not considered in the analysis. Statistical differences between mean continuous values were determined by Student's two-tailed t-test. For repeated measurements, a two-way ANOVA was used. Categorical values or continuous values that did not pass the normality test were assessed using the Mann-Whitney t-test. A value of $p<0.05$ was considered as statistically significant. Data generated by the German Mouse Clinic was analyzed using R. Tests for genotype effects were made by using Wilcoxon rank sum test and linear models depending on the assumed distribution of the parameter and the questions addressed to the data. A p-value $<$0.05 has been used as level of significance; a correction for multiple testing has not been performed.

*Study approval***.** All animal experiments were performed according to the EU Directive 2010/63/EU for animal experiments and approved by the German Animal Welfare Act (German Ministry of Agriculture, Health and Economic Cooperation), the Dutch law on animal experiment, the responsible authority of the Regierung von Oberbayern, Germany and the Institutional Ethics Committee of Universidad Autónoma de Madrid,

Madrid, Spain. Animals were socially housed under controlled conditions (22°C, 55–65% humidity, 12h light-dark cycle), and could free access water and standard laboratory chow. Adult wild-type and NOX5 knock-in male and female mice of 8-20 weeks aged were used. NOX5KI animals were compared to their respective matched WT line. The drop-out rates and outliers excluded from the analysis in all ischemia models (heart ischemia, hindlimb ischemia and stroke) are included in Table S3. A specific data point was considered as outlier if the value greatly differs from other values in a set of values, being ± the half of the mean. Post-hoc power analysis for post-stroke infarct size is included in Table S4.