

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

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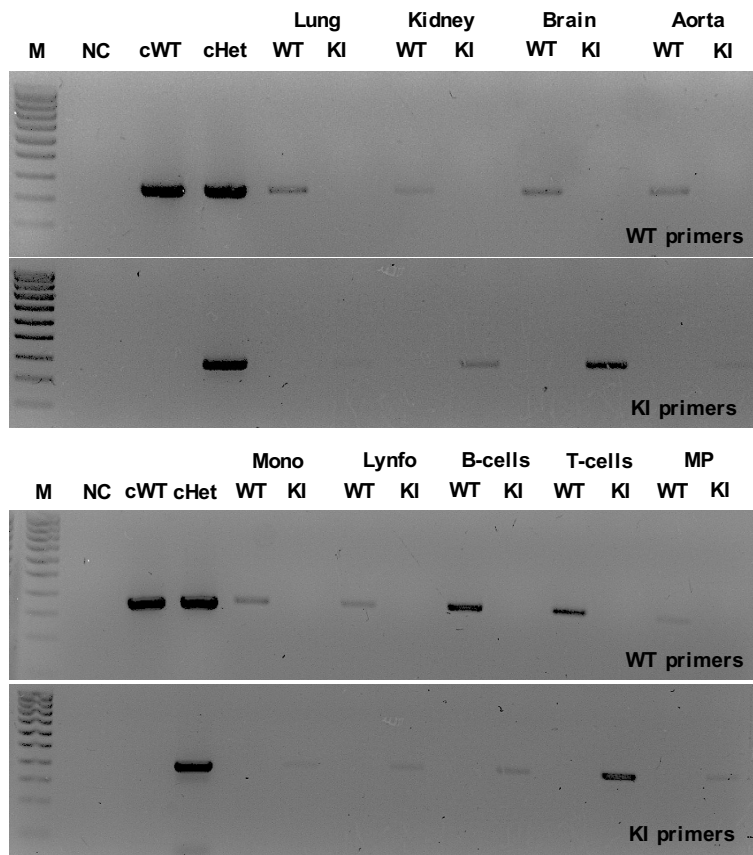
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1 **Supplementary Figures**

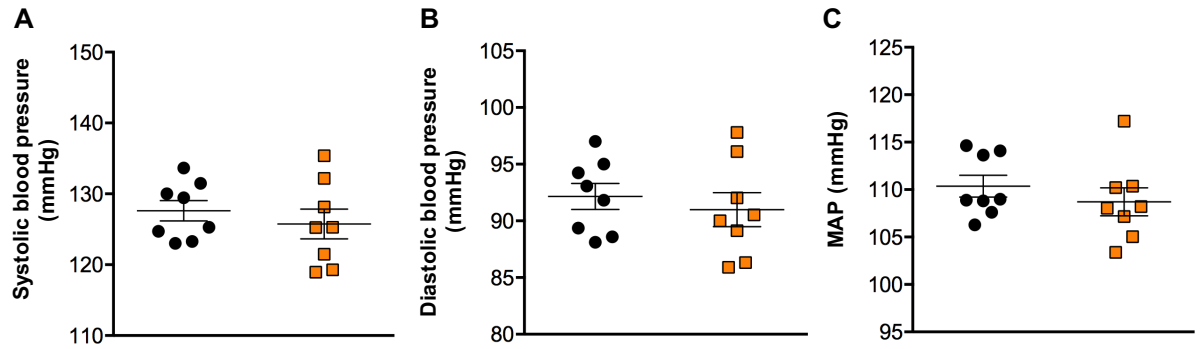


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3 **Supplemental Figure 1. NOX5KI mice genotyping.** Control (WT) and NOX5KI mice
 4 tail genomic DNA were purified and a PCR was performed to amplify the *NOX5*
 5 sequence in different tissues such as lung, kidney, brain, aorta and white blood cells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

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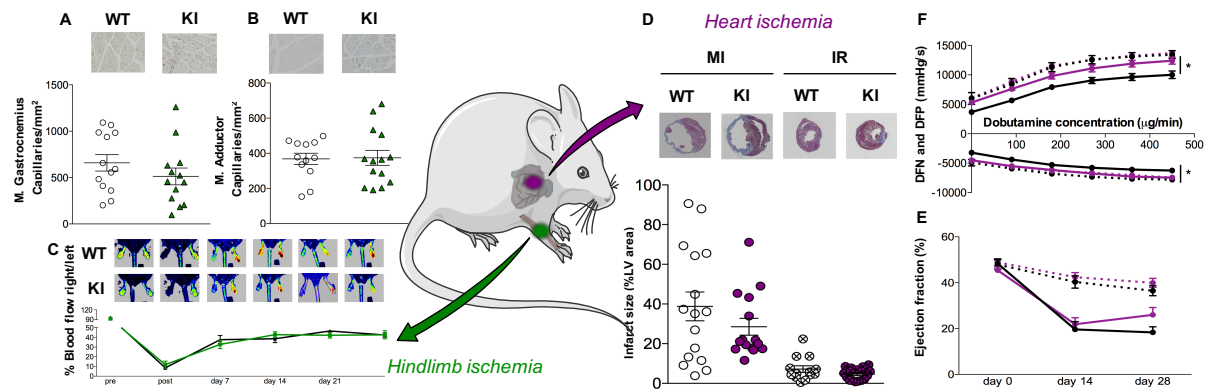
In vivo – Blood pressure measurements



1

2 **Supplemental Figure 2. No role of NOX5 in blood pressure.** (A) Systolic blood
3 pressure was not different between WT (black, n = 19) and NOX5KI mice (orange, n
4 = 20). (B) Diastolic blood pressure and (C) mean arterial pressure (MAP) did not differ
5 between WT (black, n = 19) and NOX5KI (orange, n = 20) mice during day-time (07:00-
6 18:00) or night-time (18:00-06:00).

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Supplemental Figure 3. No role of NOX5 in three different cardiovascular

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ischemic models. Capillary density (capillaries/mm³, 40X magnification) as

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measured by CD31 staining showed no difference between NOX5KI (green) and WT

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(black) mice 4-weeks after ligation of the femoral artery in (A) gastrocnemius (KI,

6

n=13; WT, n=13) (B) and adductor muscles (KI, n=14; WT, n=12).

7

(C) Blood flow restoration after ligation of the left femoral artery was not different between WT (black,

8

n = 13) and NOX5KI (green, n=10) mice.

9

(D) Infarct size (10X magnification) was not

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different between WT (black, n=16) and NOX5KI mice (purple, n=15) in the myocardial

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infarction model. Similarly, no difference was shown in the transient ischemia-

12

reperfusion heart model (hatched black, n=13; hatched purple, n=17).

13

(E) Ejection fraction decreased 2- and 4-weeks after permanent (full lines) or transient (dashed

14

lines) heart ischemia. No differences were found between WT (black, n=16; hatched,

15

n=12) and NOX5KI mice (purple, n=14; hatched, n=16).

16

(F) Functional measurements of the heart showed better contractile and relaxing properties of the NOX5KI hearts 4-

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weeks after permanent ligation (full purple lines, n=15) compared to WT (full black

18

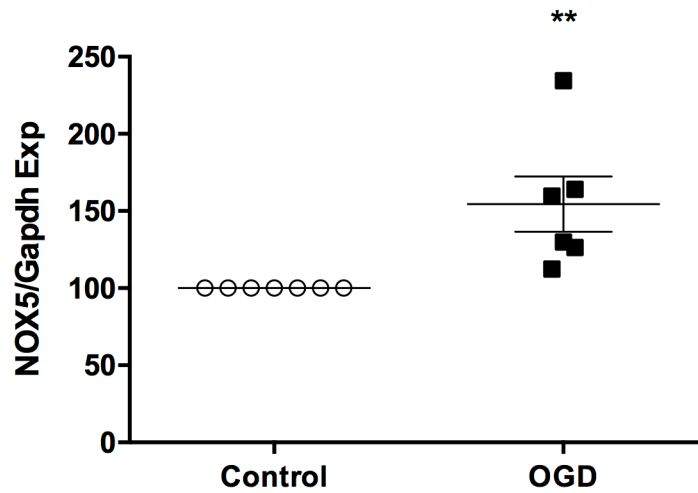
lines, n=13) (* P < 0.05, 2-tailed unpaired t test). In transient ischemia (dashed lines;

19

WT, n=12; KI, n=17), no difference was seen. Data represent as mean ± SEM in all

20

experiments.

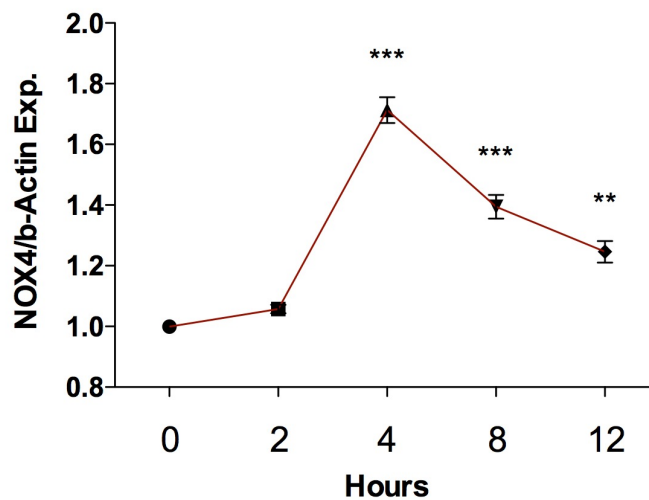


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2 **Supplemental Figure 4. *NOX5* expression in human brain microvascular**
 3 **endothelial cells (HBMECs) under ischemic conditions. *NOX5* gene expression in**
 4 HBMECs subjected to 6h of hypoxia (0.2% O₂) was significantly increased in
 5 comparison with HBMEC subjected to normoxia conditions (*P<0.05, n=5, 2-tailed
 6 unpaired t test).

7

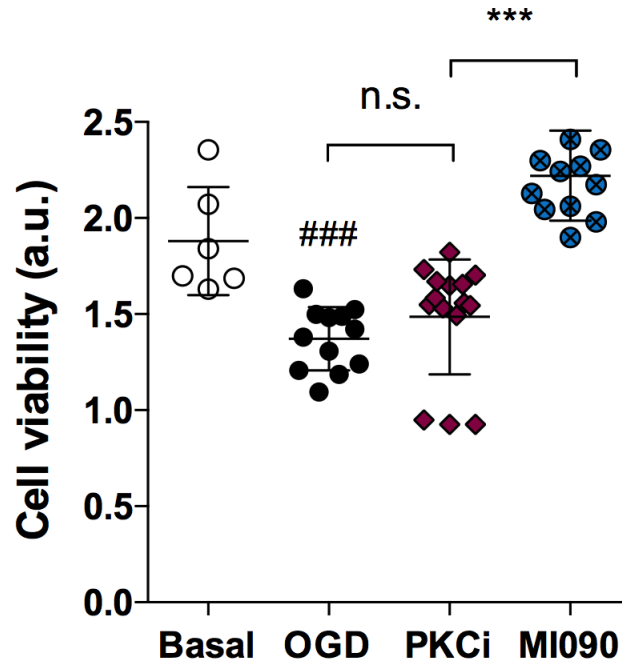
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2 **Supplemental Figure 5. NADPH oxidases 4 (NOX4) are up-regulated at different**
3 **time-points after oxygen and glucose deprivation (OGD).** Organotypic
4 hippocampal cultures (OHCs) prepared from mice hippocampal brain slices were
5 cultured for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

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

1

2 **Supplementary tables**3 **Table S1. Frequencies (mean value) of leukocyte subpopulations in peripheral blood of NOX5KI mice vs WT under**
4 **baseline conditions**

Flowcytometric measurements	Female		Male		Lineal model ⁵	
	<i>Control</i>	<i>Mutant</i>	<i>Control</i>	<i>Mutant</i>	<i>Genotype</i>	6
	n=13 mean ± sd	n=15 mean ± sd	n=9 mean ± sd	n=19 mean ± sd	p-value	7
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	8
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 ⁺ Gri ⁺	11.01 ± 3.66	10.57 ± 3.53	12.97 ± 10.27	10.96 ± 4.53	0.424	
CD45 ⁺ /NK-Gri-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 Granulocytes:(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	

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

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Flowcytometric measurements	Female		Male		p-value	p-value	Overall p-value
	<i>Control</i>	<i>Mutant</i>	<i>Control</i>	<i>Mutant</i>			
	n=13 median	n=15 median	n=9 median	n=19 median			
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 ⁺ Gri ⁺	10.6	10.2	8.93	9.28	1	0.79	0.953
CD45 ⁺ /NK-Gri-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 Granulocytes:(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

5

1 **Table S3. Study design. Animals**

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

2 *WT, wild type; KI, knock-in.*

3

1 **Table S4. Study design. Power analysis**

Infarct size (tMCAO) – NOX5 KI vs WT mice				
	<i>Mean</i>	<i>SD</i>	<i>N</i>	<i>Power for measured difference (%)</i>
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.

2
3

1 **Supplemental Methods**

2

3 *Generation of the NOX5 knock-in (KI) mouse.* Since the rodent genome naturally lacks

4 the *NADPH oxidase 5* gene, we created a new mouse line expressing the human

5 *NOX5* gene under the control of the *Tie2* promoter. The model was developed using

6 the *hypoxanthine phospho-ribosyl-transferase (Hprt)* targeted transgenic approach.

7 This locus protects transgenic constructs inserted into this region against gene

8 silencing, positional or methylation effects, and tissue-specific promoters inserted in

9 the *Hprt* locus conserve their expression patterns. Importantly, *Hprt*-deficient mice are

10 phenotypically normal. Targeted insertion at the *Hprt* locus overcomes the

11 unpredictable position effects inherent in transgenic methods relying on random

12 integration. Thus, a respective KI line allows experiments to be conducted with a single

13 mouse line, in contrast to the 3-5 transgenic lines usually required using classical

14 technology. The *Hprt* gene is localised on the X chromosome, necessitating analysis

15 of hemizygous males or homozygous females. The targeting vector was obtained by

16 subcloning of a transgenic targeting cassette containing the human *NOX5* beta gene

17 under control of the *Tie2* promoter into the *Hprt* targeting vector (GenOway, Lyon,

18 France), upstream of the human Growth Hormone polyA sequence. E14 ES cells of

19 129SV mice were transfected with the linearised targeting vector, selected with HAT

20 (hypoxanthine-aminopterin-thymidine medium) medium and resistant clones

21 screened (Southern blot) for presence of the 3' and 5' homologous recombination

22 events. Selected ES cells were injected into C57Bl6/J blastocysts and re-implanted

23 into OF1 (oestrus phase) pseudo-pregnant females, which resulted in chimeric males.

24 Chimeras were mated with WT C57Bl/6 females and agouti female offspring was

25 genotyped to confirm the presence of the recombined X chromosome. The

26 heterozygous females were then mated with transmitting male chimeras to generate

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

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

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

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

3 *Microvascular brain endothelial cell isolation:* Brain capillary endothelial cells
4 (MBCEC) from eNOX4 KO and WT mice were isolated as described in (26, 27). After
5 sacrificing the mice, forebrains were collected, meninges removed and the tissue was
6 minced and digested with a mixture of 0.75mg/ml collagenase CLS2 (Worthington,
7 USA) and 10U/mL DNase (Sigma-Aldrich, Munich, Germany) in Dulbecco's modified
8 Eagle medium (DMEM; Sigma-Aldrich, Munich, Germany) for 1h at 37°C. To remove
9 myelin, the pellet was re-suspended in BSA-DMEM (20% w/v) and centrifuged
10 (1000x g, 20 min). The pellet was re-suspended and further digested with 1mg/ml
11 collagenase-dispase (Roche, Munich, Germany) and 10U/ml SNAse in DMED for 1h
12 at 37°C. Microvascular endothelial capillaries were separated on a 33% continuous
13 Percoll gradient, collected and plated in petri-dishes coated with collagen
14 IV/fibronectin (Sigma-Aldrich, Munich, Germany). Cultures were maintained in DMEM
15 supplemented with 20% plasma-derived bovine serum (First Link, Munich, Germany),
16 50µg/ml gentamicin (Sigma-Aldrich, Munich, Germany), 2mM L-glutamine (Sigma-
17 Aldrich, Munich, Germany), 4µg/ml puromycin (Alexix GmbH, Grünberg, Germany)
18 and 1ng/ml basic fibroblast growth factor (Roche, Munich, Germany).

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

22 *Organotypic hippocampal culture (OHCs) of NOX5 KI mice.* Hippocampal brain slices
23 for cultures were obtained from brains of 7- to 10-day-old WT and NOX5 KI mice.
24 Organotypic cultures were prepared based on the methods previously described in
25 (28, 29). Briefly, pups were quickly decapitated and brains removed from the skull and

1 dissected. The hippocampus was cut into 300µm-thick slices using a Tissue Chopper
2 Mcllwain (WPI, Madrid, Spain). Then, they were separated in sterile ice-cold Hank's
3 balanced salt solution (HBSS, Biowest, Madrid, Spain) containing (in mM): glucose
4 15, CaCl₂ 1.3, KCl 5.36, NaCl 137.93, KH₂PO₄ 0.44, Na₂HPO₄ 0.34, MgCl₂ 0.49,
5 MgSO₄ 0.44, NaHCO₃ 4.1, HEPES 25, 100 U/ml penicillin, and 0.100 mg/ml
6 gentamicin. Six slices were placed on each Millicell-0.4 µm culture inserts (Millipore,
7 Madrid, Spain) within each well of a six-well culture plate. Specific neurobasal medium
8 (Invitrogen, Madrid, Spain) enriched with 10% of fetal bovine serum (Sigma-Aldrich,
9 Madrid, Spain) was used for the next 24h (1 ml/well). 24h later, B27 supplement and
10 antioxidants were added to the culture medium. Slices were in culture for 4 days before
11 inducing the oxygen and glucose deprivation (OGD) period. On day 6, inserts were
12 placed in 1 ml of OGD solution composed of (in mM): NaCl 137.93, KCl 5.36, CaCl₂
13 2, MgSO₄ 1.19, NaHCO₃ 26, KH₂PO₄ 1.18, and 2-deoxyglucose 11 (Sigma-Aldrich,
14 Madrid, Spain). The cultures were then placed in an airtight chamber (Billups and
15 Rothenberg, San Diego, USA) and exposed during 3 min to 95% N₂/5% CO₂ gas flow
16 to ensure oxygen removal. After that, the chamber was sealed for 15 min at 37°C
17 (OGD period). After 15 minutes, the cultures were returned to normal oxygen and
18 glucose concentrations for 24h (Re-Ox period).

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

1 analysis was performed using the Metamorph software version 7.0.

2 *Ca²⁺ overload in organotypic hippocampal cultures.* Hippocampal brain slices were in
3 culture 4 days as previously described. To induce Ca²⁺ overload, 10 μM of ionophore
4 A23187 (Sigma-Aldrich, Maastricht, The Netherlands) was directly added to the
5 culture medium and ROS formation was subsequently measured objective in the CA1
6 region of the hippocampus at 0, 15 and 30 min post-A23187 addition. Fluorescence
7 analysis was performed using the Metamorph software version 7.0. Different time-
8 points were selected based on previously described ROS kinetics.

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

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

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

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

20 *Motor function.* Prior to sacrifice, the mice were also scored for neurological motor
21 deficits according to the Grip Test (25). Each mouse was given a discrete value from
22 0 to 5. This score is used to evaluate motor function and coordination. The apparatus
23 is a metal rod (0.22 cm diameter, 50cm length) between two vertical supports at a
24 height of 40 cm over a flat surface. The animal is placed mid-way on this rod and is
25 rated according to the following system: Score 0, falls off; 1, hangs on to string by one

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

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

8 *Oxidative stress: DHE staining.* The presence of ROS was determined using
9 dihydroethidium, stock solution 2 mM (Sigma-Aldrich, Würzburg, Germany) staining in
10 coronal brain sections taken from identical regions (-0.5mm from bregma) of the
11 different animal groups. Briefly, frozen sections were incubated in 2 μ M DHE for 30
12 minutes at 37°C, washed three times with PBS and incubated with Hoechst (Hoechst
13 33342, Sigma-Aldrich, Würzburg, Germany) 2 ng/ml for 10 min. All sections were
14 analyzed and acquired with a Nikon Eclipse 50i microscope equipped with the DS-U3
15 DS camera control unit. The relative pixel intensity was measured in identical regions
16 with NIS- Elements software (Nikon, Tokyo, Japan). Digital images were processed
17 using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

18 *Heart ischemia: Myocardial infarction and ischemia reperfusion.* Mice aged 8-16
19 weeks were subjected to permanent or transient ligation of the left descending
20 coronary artery. After administration of an analgesic (buprenorphine s.c. 0.05mg/kg,
21 Temgesic, Schering-Plough, USA), mice were anaesthetized with isoflurane (Abbott
22 forene Isoflurane, Maastricht, The Netherlands) 4-5% in air and intubated per orally
23 with a stainless-steel tube. The tube was connected to a respirator (rodent ventilator
24 Microvent type 845, Hugo Sachs Electronic, Germany), set at a stroke volume of
25 250 μ L and a rate of 210 strokes/min. Anaesthesia was then maintained with 2-3%

1 isoflurane in air via a vaporizer (Univentor, UNO Roestvaststaal BV, Germany)
2 connected to the respirator. The mouse was placed on a heating pad (UNO
3 temperature control unit, UNO Roestvaststaal BV, Germany) and body temperature
4 was monitored using a rectal probe and maintained at 37.0°C using a feedback-
5 controlled infrared light. During surgery, an ECG was recorded with IDEEQ software
6 (IDEE, Maastricht University, The Netherlands). A left thoracotomy was performed to
7 expose the heart. Then, the left descending coronary artery (LAD) was ligated with a
8 6-0 polypropylene suture (Surgipo, Chicago, IL, USA), just proximal to its main
9 branching point. The suture was tied around a 3 mm-long polyethylene tube (PE-10)
10 to induce ischemia. Ischemia was assessed by a discolouration of the tissue (from red
11 to pink-white) and ST-elevation on ECG. After 45 minutes, the blood flow was re-
12 established by removal of the polyethylene-tube. The occurrence of reperfusion was
13 assessed by the colour of the tissue turning red again. For the myocardial infarct
14 model, the LAD was ligated with a 6-0 polypropylene suture permanently. The chest
15 was closed with 5-0 silk sutures (Ethicon, USA). The animals were weaned from the
16 respirator and the endotracheal tube was removed, once the mice breathed
17 spontaneously. After surgery, mice were allowed to recover at thermoneutral
18 temperature (28°C). In the morning and afternoon of the day after, s.c. 0.05 mg/kg
19 buprenorphine hydrochloride was repeated to relieve pain.

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

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

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

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

1 Qwin pro v3.5.1 software. For each animal, three random pictures were taken per
2 muscle sample and the amount of capillaries is expressed as number per mm².

3 *Human brain microvascular endothelial cells (HBMECs) subjected to hypoxia.* Primary
4 culture HBMEC (cat. N. ACBRI_376_V, Cell systems, USA) between passage 3 and
5 9 were cultured to approximately 95% confluence using recommended cell medium
6 (EGM-2 MV BulletKit, Lonza, Maastricht, The Netherlands) enriched with 5% fetal
7 bovine serum (FBS; Sigma-Aldrich, Maastricht, The Netherlands) before starting the
8 experiment. For hypoxia studies, HBMECs were always seeded at specific cell density
9 (6×10^4 cells/ml) in 12 wells-plate and incubated during 24h at 37°C. Then, cell medium
10 was replaced with non-FBS enriched medium (2 ml/well) following by 6h of hypoxia
11 (94,8% N₂, 0.2% O₂ and 5% CO₂) at 37°C using hypoxia workstations (Ruskin Invivo2
12 400 station, Maastricht, The Netherlands). The hypoxia period was followed by 24h of
13 re-oxygenation at 37°C, enriched medium and normoxia conditions (75% N₂, 20% O₂
14 and 5% CO₂) in the presence or absence of treatment. All culture surfaces were pre-
15 treated with fibronectin solution (1:100 in PBS, Sigma-Aldrich, Maastricht, The
16 Netherlands).

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

20 *Assessment of cell permeability in HBMECs.* 2×10^4 HBMECs were seeded and
21 incubated during 24h on Transwell inserts (collagen-coated Transwell Pore Polyester
22 Membrane Insert; pore size = 3.0 µm, Corning, Maastricht, The Netherlands) before
23 inducing 6h of hypoxia (94,8% N₂, 0.2% O₂ and 5% CO₂) followed by 24h re-
24 oxygenation period in presence/absence of early or late treatment. The Evans Blue
25 dye (Sigma-Aldrich, Madrid, The Netherlands) was used to assess cell permeability

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

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

21 *Study approval.* All animal experiments were performed according to the EU Directive
22 2010/63/EU for animal experiments and approved by the German Animal Welfare Act
23 (German Ministry of Agriculture, Health and Economic Cooperation), the Dutch law on
24 animal experiment, the responsible authority of the Regierung von Oberbayern,
25 Germany and the Institutional Ethics Committee of Universidad Autónoma de Madrid,

1 Madrid, Spain. Animals were socially housed under controlled conditions (22°C, 55–
2 65% humidity, 12h light-dark cycle), and could free access water and standard
3 laboratory chow. Adult wild-type and NOX5 knock-in male and female mice of 8-20
4 weeks aged were used. NOX5KI animals were compared to their respective matched
5 WT line. The drop-out rates and outliers excluded from the analysis in all ischemia
6 models (heart ischemia, hindlimb ischemia and stroke) are included in Table S3. A
7 specific data point was considered as outlier if the value greatly differs from other
8 values in a set of values, being \pm the half of the mean. Post-hoc power analysis for
9 post-stroke infarct size is included in Table S4.

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