NOX4-dependent neuronal autotoxicity and BBB breakdown explain the superior sensitivity of the brain to ischemic damage

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Ischemic injury represents the most frequent cause of death and disability, and it remains unclear why, of all body organs, the brain is most sensitive to hypoxia. In many tissues, type 4 NADPH oxidase is induced upon ischemia or hypoxia, converting oxygen to reactive oxygen species. Here, we show in mouse models of ischemia in the heart, brain, and hindlimb that only in the brain does NADPH oxidase 4 (NOX4) lead to ischemic damage. We explain this distinct cellular distribution pattern through cell-specific knockouts. Endothelial NOX4 breaks down the BBB, while neuronal NOX4 leads to neuronal autotoxicity. Vascular smooth muscle NOX4, the common denominator of ischemia within all ischemic organs, played no apparent role. The direct neuroprotective potential of pharmacological NOX4 inhibition was confirmed in an ex vivo model, free of vascular and BBB components. Our results demonstrate that the heightened sensitivity of the brain to ischemic damage is due to an organ-specific role of NOX4 in blood-brain-barrier endothelial cells and neurons. This mechanism is conserved in at least two rodents and humans, making NOX4 a prime target for a first-in-class mechanism-based, cytoprotective therapy in the unmet high medical need indication of ischemic stroke.

Significance

Why the brain is uniquely sensitive to hypoxia and which cells are involved is incompletely understood. Here we identify that, upon ischemic stroke, in endothelial cells and neurons the reactive oxygen-forming NADPH oxidase 4 (NOX4) causes breakdown of the BBB and neuronal cell death. This mechanism is unique to the brain and not found in other forms of ischemia in the body. Genetic deletion of either cell type (endothelial or neuronal) or pharmacological inhibition of NOX4 leads to a significant reduction of infarct volume and direct neuroprotection. This mechanism explains the unique vulnerability of the hypoxic brain compared with other organs and provides a clear rationale for first-in-class neuroprotective therapies in stroke.


The authors declare no conflict of interest.

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PHARMACOLOGY
permanent occlusion of the middle cerebral artery in Nox4 KO mice showed the same infarct size reduction as in the transient occlusion (9).

To investigate the role of NOX4 in hindlimb ischemia, which mimics human peripheral artery disease, sexually mature adult Nox4 KO and WT mice from 12 to 16 wk old were subjected to permanent ligation of the femoral artery. Angiogenesis (sprouting of new capillaries) occurs as a response to the ischemic muscle damage, mainly in the distal calf muscles (13), and was assessed via the capillary density marker, CD31, at day 28 as reported previously (11) and well within the reported range of 14 and 35 d postligation (15). No significant differences were observed in the capillary density marker, CD31, at day 28 as reported previously (11) and well within the reported range of 14 and 35 d postligation (15). Thus, although NOX4 was up-regulated in all three ischemic models, deletion of Nox4 KO mice. Thus, although NOX4 was up-regulated in all three ischemic models, deletion of Nox4 did not modify outcomes after ischemia of the heart nor of the hindlimb. Deletion of Nox4, however, conveyed a major a protective in stroke, indicating a surprising highly organ-specific role of NOX4.

As a possible explanations for this, the blood–brain barrier (BBB) is a unique feature of the brain versus heart and skeletal muscle and, in ischemic brain, proinflammatory immune cells (17) and NOX4 impairs myogenic tone (15). We therefore investigated the contribution of both vascular cell types, endothelial and vascular smooth muscle. Moreover, as some neurons were also NOX4 immunopositive (9), we also investigated neuron-specific Nox4 KO mice (Fig. 2A).

**Cell-Specific Nox4 KO Mouse Generation and Validation.** Endothelial cell-specific Nox4 KO mice were generated by crossing Nox4 floxed homozygous female mice with Tie2-Cre+ (endothelial cell-specific promoter) male mice. Neuronal and SMC-specific Nox4 KO mice were generated using inducible CaMKII–Cre+ (17) and SMMHC–Cre+ (18) mice, respectively. While neuronal (nKO) and SMC NOX4 KO (sKO) are tamoxifen-inducible mouse lines, endothelial NOX4 KO (eKO) mice bear a constitutive deletion. Therefore, both SMC (sWT) and neuronal WT (nWT) require tamoxifen injections before studying their KO phenotype, and consequently both share the tamoxifen-treated WT line as a control (n/sWT) (SI Appendix, Fig. S3). Cointaining of NOX4 and CD31 showed the cell-specific absence of the NOX4 protein in endothelial and neuronal (eKO and nKO) in the brain of endothelial Nox4 KO (eKO in the first row, Fig. 2B, second row) but not in SMC-specific Nox4 KO (sKO) (Fig. 2B, Left, fourth row). Using a similar cointaining approach of NOX4 and NeuN, no NOX4 could be detected in neurons of the neuronal Nox4 KO (nKO) mice (Fig. 2B, Left, third row). As no SMC could be stained in the brain, for control purposes, carotid tissue was used to confirm that SMC of the sKO indeed do not express NOX4 (Fig. 2B, Right). Collectively, this confirmed three cell-specific Nox4 KO mouse lines: eKO (constitutive), nKO (inducible), and sKO (inducible) (SI Appendix, Fig. S3). The Tie2 promoter is also active in bone marrow. However, Nox4 expression was beyond the detection limit in both WT and Tie2-Cre+ mice while β-actin (internal control) and NOS2 signals, the major immune cell NOX isoform, were strong and similar both in WT and Tie2-Cre+ mice. These data suggest that macrophages/bone marrow cells would not contribute to an eKO phenotype (SI Appendix, Table S1).

**Endothelial and Neuronal, but Not Smooth Muscle, NOX4 Deficiency Improves Stroke Outcome.** To examine the poststroke impact of cell-specific eKO, sKO, and nKO of Nox4, 8- to 12-wk-old mice were subjected to 1 h of mTCAO followed by 24 h of reperfusion. TTC-stained brain sections showed a significant reduction of infarct volume on day 1 after surgery in eKO and nKO compared with WT mice, but not in sKO mice (Fig. 3A). As a clinical parameter, two independent neurological outcome parameters were assessed, which measure global neurological and motor
impairments, i.e., the Bederson score (Fig. 3B) and the Grip test (Fig. 3C). Both outcome scores were significantly improved in eKO and nKO but not in sKO mice, the latter showing even a slightly deleterious outcome in the Grip test (Fig. 3C). These data suggest a contribution of endothelial and neuronal but not SMC NOX4 in stroke.

Differential Roles of Endothelial and Neuronal NOX4 in BBB Breakdown and Neurotoxicity. Increased vascular permeability and subsequent neurotoxicity are considered key contributors to poststroke pathophysiology (19). Since the localizations of NOX4 in mice (Fig. 2B) and humans (19) match these two pathomechanisms, we hypothesized that NOX4 may contribute to both of them.

First, we assessed the role of NOX4 in BBB disruption by functional quantification of poststroke edema formation using extravasation of an extravascular tracer. Evans blue, into the brain parenchyma. Evans Blue extravasation was significantly reduced in brains from eKO but not nKO mice, indicating a specific role of eNOX4 in poststroke BBB stability (Fig. 4A). Second, we quantified neuronal cell death by costaining for the neuronal marker NeuN and a TUNEL detection kit. Apoptotic neurons were reduced in nKO (Fig. 4B) but not eKO mice (Fig. 4B). Necrotic cells were also assessed in both eKO and nKO mice, showing similar results, no effect in eKO, and reduction in nKO (SI Appendix, Fig. S4). Autophagy may be an alternative pathomechanism in brain ischemia (20). Indeed, in stroke eKO versus WT mice the autophagy marker, beclin-1, was significantly reduced (SI Appendix, Fig. S5). Thus, BBB protection and smaller infarct in eNOX4 KO are most likely linked to less autophagic stress and subsequently a reduction in cell demise (21), while nNOX4 KO mice showed direct neuroprotection via an antiapoptotic mechanism.

To test the link of both observations to the enzymatic activity of NOX4, i.e., ROS formation, we measured oxidative stress using dihydroethidium staining of cryosections. ROS generation was significantly reduced in the infarcted brains of both nKO and eKO mice compared with their corresponding controls (Fig. 4C). Thus, ROS formation appears to be a common denominator of both eNOX4 and nNOX4.

Neuroprotective Effect of Neuronal NOX4 Inhibition Is Confirmed by in Vivo and in Vitro Approaches in a Second Species. The stroke therapy academic industry roundtable (STAIR) criteria recommend that any result should be corroborated in another animal species (www.thestair.org) (22). Until now only mice have been used to show that NOX4 inhibition reduces infarct volume and neurological deficits following cerebral ischemia (9). To further validate the role of NOX4 in stroke pathophysiology and outcome, we extended our studies by using both rat models (Fig. S4). We generated global Nox4 KO rats (SI Appendix, Fig. S6) and confirmed the absence of Nox4 DNA markers by genotyping (SI Appendix, Fig. S7) and brain, kidney, and lung mRNA markers by qPCR including β-actin as internal control (SI Appendix, Table S2). No phenotypic differences were observed in the Nox4 KO rat compared with its respective WT. Nox4 KO rats were then subjected to 90 min of MCAO followed by 24 h of reperfusion. Consistent with our in vivo observations in the mouse model, Nox4 KO rats showed significantly smaller infarct volumes compared with the WT littermates (Fig. 5B). Importantly, replicating the protective effect of Nox4 deletion in a second species further strengthens the attractive hypothesis of NOX4 inhibition as a neuroprotective therapeutic approach for stroke patients.

We next used an in vitro rat hippocampal brain slice model mimicking ischemic conditions by oxygen and glucose deprivation (OGD), which allowed us (i) to corroborate our findings from the in vivo studies, using a translationally more relevant pharmacological approach and (ii) to further analyze nNOX4 function independently of any functional vascular component. Hippocampal brain slices were subjected to 15 min to OGD followed by 2 h of reoxygenation in the presence or absence of the NOX inhibitors GKT136901 (0.1 μM) or VAS2870 (10 μM). At the end of the experiment, hippocampal cell death was assessed by the MTT assay, and we observed that both NOX4 inhibitors were neuroprotective after an OGD period (Fig. 5C). Similarly, oxidative stress measured by the H2DCFDA probe was significantly reduced after 2 h of reoxygenation under NOX inhibitor treatment (Fig. 5D). To support these findings, hippocampal brain slices from Nox4 KO mice were subjected to OGD for 15 min followed by a 2-h reoxygenation period. As expected, cell death (SI Appendix, Fig. S8) and ROS formation (SI Appendix, Fig. S8D) were significantly reduced in Nox4 KO mice compared with their respective WT littermates.

Once both rodent species (mouse and rat) showed direct neuroprotection and reduction of brain damage poststroke, we extended our findings to a human in vitro model of BBB. Primary cultures of human brain microvascular endothelial cells (HBMEC) were subjected to 6 h of hypoxia followed by 24 h of reoxygenation under NOX inhibitor treatment (Fig. 5D). To support these findings, hippocampal brain slices from Nox4 KO mice were subjected to OGD for 15 min followed by a 2-h reoxygenation period. As expected, cell death (SI Appendix, Fig. S8) and ROS formation (SI Appendix, Fig. S8D) were significantly reduced in Nox4 KO mice compared with their respective WT littermates.

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dextran tracer (3 and 70 kDa) (SI Appendix, Fig. S10A and B) and Evans Blue extravasation (SI Appendix, Fig. S10C) through a monolayer of HBMEC.

Using a combined preclinical meta-analysis, we previously established that NOX1 plays no role in brain ischemia (12). Nevertheless, GKT136901 has been described as a NOX1/NOX4 inhibitor (23) and may have acted also via NOX1. To test this possibility, Nox4 KO mice were subjected to 1 h tMCAO and treated 1 h poststroke with GKT136901 (10 mg/kg), showing no additional effect over nontreated Nox4 KO animals (SI Appendix, Fig. S11). Therefore, these data suggest that NOX1 plays no role in the action of GKT136901 and that NOX4-driven neurotoxicity can be pharmacologically prevented using isoform-specific NOX inhibitors that inhibit massive ROS production and oxidative damage. Hence, pharmacological modulation of NOX4 could lead to direct neuroprotection and improvement of BBB stability.

**Discussion**

Here we elucidate one mechanism: why, of all organs, the brain has the highest sensitivity to hypoxia/ischemia. The induction of NOX4, a primary source of ROS, in many tissues during hypoxia (9–11) suggested that NOX4 may be a uniform target for post-ischemic cytoprotective therapies. Surprisingly, however, we found the role of NOX4 in ischemia to be highly specific to the brain, i.e., in ischemic brain stroke, and not to be involved in lung ischemia (24), myocardial IR, or hindlimb ischemia. We have previously shown that the predominant localizations of NOX4 in the mouse and human brain, pre- and poststroke, are in endothelial cells and neurons (9). At the mRNA level, other cells have been described to express NOX4, which, however, does not translate to detectable levels at the protein level. By examining cell-specific Nox4 KO mice, we provide clear evidence on how endothelial and neuronal Nox4 have a major impact in brain...
vulnerability to ischemia. However, we cannot exclude that Nox4 minor expression in other cells may also contribute, in smaller percentages, to the observed phenotype. For example, pericytes have been suggested to play an important role in the formation and maintenance of the BBB, leading to a detrimental role in acute ischemia (25). Moreover, Nox4 has been recently considered as the major source of ROS in human pericytes (26). However, the fact that the endothelial and neuronal Nox4 KO mice add up completely to the global Nox4 KO phenotype (26) suggests that in our stroke model pericyte contribution is indeed rather minor and endothelial and neuronal cells constitute the main sources of NOX4 relevant to stroke damage.

One key explanation for the brain-specific role of NOX4 appears to reside in the BBB, which differentiates the brain from the heart and other organs (24). We identify endothelial NOX4, but not vascular smooth muscle nor neuronal NOX4, as the key source of ROS triggering to breakdown upon an ischemic stroke. In heart and vascular periphery such leakage and edema may also occur but is apparently less detrimental (25). Indeed, we find that NOX4 inhibition prevents BBB leakage in a human BBB in vitro model. It will be worth exploring whether in other organs with a blood–tissue barrier, e.g., the placenta (26) or mammary glands, a similar pathological relevance during ischemia or hypoxia is observed.

A second unexpected mechanism is that NOX4 can cause direct neuronal autotoxicity. Previously, NOX4 was thought to be primarily relevant in the vasculature. Neuronal autotoxicity in stroke, also termed excitoxicity, was previously ascribed primarily to nitric oxide overproduction (27) and neurotrauma (28). However, both in vivo and also importantly in an ex vivo brain slice model free of BBB effects, neuronal NOX4 was clearly a major contributor to cellular autotoxicity upon ischemia or hypoxia. Pharmacological inhibition of NOX4 was also neuroprotective, providing proof-of-concept for the druggability of this component of NOX4's actions as well.

Both cellular mechanisms explain why NOX4 inhibition is such a powerful experimental therapeutic approach in stroke (9). It directly prevents two damage-trigger mechanisms: the breakdown of the BBB and the subsequent neuronal autotoxicity. To better estimate whether these mouse data may be translatable into the clinic as a first-in-class cytoprotective therapy in stroke, future experiments should confirm NOX4's role in a second species and then translate it to humans. It is therefore reassuring that the essential role of NOX4 in stroke is preserved in a second rodent species using a Nox4 KO rat and by pharmacological intervention in a human brain microvascular endothelial cell model. Subsequent studies in large-animal stroke models and preparation for a clinical trial are ongoing (European Research Council—Proof of Concept Project 737586 SAVEBRAIN).

This study cannot elucidate why the substantial induction of NOX4 and subsequent ROS formation in heart and hindlimb and other organs (29) does not lead to tissue damage. However, this is reminiscent of the induction of NOX4 in the lung during hypoxia (9) and ischemia (26), which is also not detrimental. Previous studies looking at the short-term effects (after 24 h) showed either no (30) or a detrimental role (31) of NOX4 in heart ischemia. We are aware that Nox4 has been proposed to be one of the major sources of ROS in failing hearts (26), playing a key role in cardiac failure (32) and mitochondrial growth (33) and dysfunction in heart tissue (34). However, our findings do not indicate a short-term effect in heart ischemia. Likewise, looking at long-term outcomes in hindlimb and MI models, where the assessment was performed 4 wk after surgery, we detected no significant effect. With respect to the hindlimb model of ischemia, a variation of this model using an artery excision technique, which results in more inflammation and surrounding tissue damage, NOX4 is protective (11, 35, 36), which may involve NOX4’s anti-inflammatory role (36, 37). Using a pure ischemic hindlimb model, different KO strategy, and anesthesia, we find no role for NOX4.

In conclusion, we elucidate the superior sensitivity of the brain compared with other organs to ischemic damage at the cellular level by NOX4-dependent neuronal autotoxicity and BBB breakdown. Our findings also provide a clear rational for further development of NOX4 inhibitors as a first-in-class neuroprotective strategy in stroke.

Materials and Methods

Detailed methods are provided in SI Appendix, SI Materials and Methods.

Animals. All animal experiments were approved by local state authorities (Regierung von Unterfranken) as well as by the German Animal Welfare Act, the Dutch law on animal experiments, and the Ethics Committee of the Faculty of Medicine, Universidad Autonoma de Madrid (Madrid).

Cell-Specific Nox4 KO Mice Generation. Constitutive Nox4 KO mice and floxed Nox4 mice were generated as described (9). To generate endothelial-specific Nox4 KO (eNox4 KO) mice, female mice were bred with male mice that express the Cre recombinase gene under control of the endothelial-cell-specific Tie2 promoter (18). During breeding, only males that bear the Cre gene were selected for future breeding rounds, while females were not allowed to bear the Cre. For experiments Cre/− (eNox4 KO) males were used. SMC-specific and neuron-specific Nox4 KO mice were generated in an analogous way using SMMHC-CreERT2 mice (18) and CamKII Cre (17) mice, respectively.

Stroke Surgery (tMCAO Model). The model was previously established as described for mice (9) and rat (38) surgery.

Brain Infarct Volume Measurements. The ischemic lesion was measured 24 h after MCAO using TTC staining (38).

Murine Model of Hindlimb Ischemia. The right femoral artery was permanently ligated. A suture (5-0 silk) was placed around the femoral artery in
between the branching of the artery epigastria 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 polyorb suture.

MI and IR of the Heart Models. Using a left thoracotomy, the left descending coronary artery was ligated with a 6-0 polypropylene suture permanently for MI. For the transient ischemia of 45 min, a small polyethylene tube was inserted under the ligature, compressing the coronary artery, which was then removed after the ischemic period.

Hippocampal Brain Slices. In vitro damage caused by oxygen–glucose deprivation/reoxygenation and the protection elicited by NOX inhibitors was studied in rat hippocampal slices. Brains from 2- to 3-mo-old male Sprague-Dawley rats (250–300 g) or Nox4 KO mice (2–3 mo) were isolated and submitted to OGD as previously described (39).

HBMEC Culture Subjected to Hypoxia. HBMECs (Cell Systems) were cultured to ~90% confluence. Cell medium was replaced for non-FBS–containing medium (2 mL/well) following 6 h of hypoxia (94.8% N2, 0.2% O2, and 5% CO2). The hypoxia period was followed by 24 h of reperfusion in the presence or absence of 1 μM GKT136901 (Genkyotex) as treatment.

Statistics. All mice and in vitro data are expressed as mean ± SEM. Rat in vivo experiments are expressed as mean ± SD. Using the GraphPad Prism 5.0 software package statistical differences were determined by Student’s two-tailed t test and Mann–Whitney test experiments. For repeated measurements, a two-way ANOVA was used. Statistical comparisons between groups were performed using one-way ANOVA. The number of animals necessary to detect a standardized effect size on infant cardiac volumes ≥0.2 was determined via a priori sample-size calculation. P values < 0.05 were considered statistically significant.

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Supplementary Material: NOX4-dependent neuronal autotoxicity and blood-brain barrier breakdown explain the superior sensitivity of the brain to ischemic damage

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Supplementary Materials and Methods

Animals

All animal experiments were approved by local state authorities (Regierung von Unterfranken), comply with the ARRIVE guidelines and are carried out in accordance with the EU Directive 2010/63/EU for animal experiments as well as the German Animal Welfare Act (German Ministry of Agriculture, Health and Economic Cooperation), the Dutch law on animal experiments and were approved by the Ethics Committee of Faculty of Medicine, Universidad Autónoma de Madrid (Madrid, Spain). Animals were housed under controlled conditions (22°C, 55–65% humidity, 12h light-dark cycle), and were allowed free access to water and standard laboratory chow. Male and female mice aged 8-16 weeks and adult rats (>12 weeks) were used. The Nox4 KO animals were compared to their respective matched WT’s.

NOX4 expression in different ischemia models

Muscle from the lower leg from wild type mice subjected to a permanent ligation of the femoral artery and heart apex after occlusion of the left descending coronary artery were collected together with tissue samples from matched non-ischemic mice. Brain tissue from stroked and non-stroked brain areas were collected and snap-frozen. Similarly, human brain microvascular endothelial cells were collected following the hypoxia period. After homogenization, total mRNA was prepared by using the TRI Reagent® (Sigma-Aldrich, The Netherlands) and was quantified spectrophotometrically. 0.08 µg of total mRNA was reverse transcribed to cDNA with the High Capacity Reverse Transcription Kit (Applied Biosystems, The Netherlands) according to the manufacturer’s protocol. mRNA levels of Nox4 were quantified by using the fluorescent Taqman® technology. Cyplophilin and Gapdh were used as reference genes for the in vivo and in vitro models respectively. We used TaqMan®
gene expression arrays (TaqMan® Universal PCR Master Mix, ThermoFisher Scientific, The Netherlands) specific for mouse: Nox4 (Mm00479246_m1, ThermoFisher Scientific, The Netherlands), mouse Cyclophilin (Mm02342430_g1, ThermoFisher Scientific, The Netherlands), Gapdh (Mm99999915_g1, ThermoFisher Scientific, The Netherlands) and human: Nox4 (Hs00418353_m1, ThermoFisher Scientific, The Netherlands), Gapdh (Hs02758991_g1, ThermoFisher Scientific, The Netherlands). Water controls were included to ensure specificity and the comparative ΔΔCt method was used for relative quantification of gene expression.

Cell specific Nox4 KO mice generation

Constitutive Nox4 KO mice and floxed Nox4 mice were generated as described (1). To generate endothelial-specific Nox4 KO (eNox4 KO) mice, female mice (homozygous for the floxed NOX4 gene) were bred with male mice (C57Bl6 strain background) that express the Cre recombinase gene under control of the endothelial-cell specific Tie2 promoter (2). As the Cre is located on the X chromosome, all males used for this breeding were hemizygous for the Cre gene (Cre<sup>+</sup>/y). During breeding, only males that bear the Cre gene were selected for future breeding rounds, while females were not allowed to bear the Cre. For experiments Cre positive (eNox4 KO) males were used. Smooth muscle cell-specific and neuron specific Nox4 KO mice were generated in an analogous way using SMMHC-CreERT2 mice (2) and CamKII Cre (3) (EMMA ID number 01153) mice respectively. Deletion of NOX4 in SMC and neurons was induced using Tamoxifen as described (4). For tMCAO, male 8-10 weeks old Cre positive mice treated with tamoxifen (sNox4 KO and nNox4 KO) were used. For all three lines Cre negative NOX4FF mice were used as controls (WT), with the WT groups for the SMC and neuron specific lines also being injected with tamoxifen.
Verification of NOX4 deficiency in eKO, nKO and sKO mice

Histology and immunohistochemistry were performed according to standard procedures (5). The cell-specific Nox4 KO mice validation was performed on formalin fixed paraffin embedded tissue (brain or aorta) that was pre-treated with Proteinase K prior to antibody incubation. For specific staining, the following antibodies were used: pAb anti -CD31 (Biorad, MCA2388), NOX4 (kindly provided by A.M. Shah, King’s College London British Heart Foundation Centre, London) and anti-NeuN (Millipore, MAB337). DNA was visualized with Hoechst and sections were coverslipped using Aqua Poly Mount. All sections were analyzed with a Nikon Eclipse 50 microscope equipped with the DS-U3 DS camera control unit and NIS- Elements software (Nikon, Tokyo, Japan).

NOX4 expression in bone marrow flushes

Bone marrow samples from endothelial NOX4 KO and their respective WT mice were collected. mRNA isolation and cDNA preparation were prepared as previously described. mRNA levels of Nox4 were quantified by using the fluorescent Taqman® technology. β-actin were used as reference gene. We used TaqMan® gene expression arrays (TaqMan® Universal PCR Master Mix, ThermoFisher Scientific, The Netherlands) specific for mouse: Nox4 (Mm00479246_m1, ThermoFisher Scientific, The Netherlands), Nox2 (Mm1287743_m1, ThermoFisher Scientific, The Netherlands) and β-actin (Mm02619580_g1, ThermoFisher Scientific, The Netherlands). Water controls were included to ensure specificity.

Nox4 KO rat generation

Nox4 KO rats were generated at Sage Labs using the CompoZr Zinc Finger Nuclease technology. The E14-15 domain (2.3-2.4 Kb) of the Nox4 gene was removed in a WKY background rat. Zinc-Finger Nucleases (ZFNs) were coupled with a FOK1
endonuclease and designed to recognize and cleave the specific NOX4 sequence producing sequence-specific double-strand breaks that are repaired by error-prone non-homologous end joining (NHEJ) or high-fidelity homologous recombination (HR).

The Nox4 KO rat was generated by introducing variable genomic deletions that result in a frameshift within the open reading frame. The frameshifts often result in the introduction of a premature stop codon. When the premature stop codon occurs before the last exon, the transcript is likely degraded via nonsense mediated decay pathway and little or no protein is expressed. Resulting animals were screened for mutations and complete genomic sequencing was performed.

**Nox4 KO rat genotyping**

DNA isolation and PCR from rat tail cuts was performed using the Quanta Bioscences AccuStart II Rat/Mouse genotyping kit (VWR cat no. 95135-500) according to the instructions from the manufacturer with the following primers: forward Int13 Cel1 5'-TGTCTGCCAGAGCATTCACTA-3', reverse Int13 Cel1 5'-CAAATGGACTTCCAAATGGG-3' and reverse Int15 Cel1 5'-CTTCTGCAGTCTACCCTGGC-3'. A 2% Agarose gel was used, running for 45-50 minutes. Expected PCR products are 383bp for WT and 300bp for KO.

**Verification of NOX4 deficiency in Nox4 KO rats**

Brain, kidney and lung tissue were collected from WT and Nox4 KO rats. mRNA isolation and cDNA preparation were prepared as previously described. mRNA levels of Nox1, Nox2 and Nox4 in brain, kidney and lung were quantified by using the fluorescent Taqman® technology. We used TaqMan® gene expression arrays (TaqMan® Universal PCR Master Mix, ThermoFisher Scientific, The Netherlands) specific for rat Nox1 (Rn00586652_m1, ThermoFisher Scientific, The Netherlands), Nox2 (Rn00576710_m1, ThermoFisher Scientific, The Netherlands) and Nox4.
(Rn01506793_m1, ThermoFisher Scientific, The Netherlands). Water controls were included to ensure specificity and the comparative \( \Delta \text{Ct} \) method was used for relative quantification of gene expression.

**Stroke surgery (tMCAO model)**

The model has previously been established as described in (1) for mice and (6) rat surgery. Animals were anesthetized with isoflurane (2-2.5% 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), 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 and 4.0 nylon monofilament (40SP, Doccol Corporation, Sharon, MA, USA) for rats 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 1 hour after occlusion by monofilament removal in mice and 1.5h for rat experiments. After the surgery, wounds were carefully sutured and animals were allowed to recover from surgery in a temperature-controlled cupboard. Animals were excluded from the stroke analysis, if animals died before the predefined experimental end point, if an intracerebral hemorrhage occurred, if the
animal lost more than 20% of body weight or if the animal scored 0 on the Bederson score (SI Appendix, Table S3).

**Treatment with NOX inhibitor: GKT136901**

GKT136901 was dissolved in a mixture of DMSO/water in a ratio of 1/99. GKT136901 (10 mg/kg) or vehicle (DMSO/water in a ratio of 1/99) were injected i.p. 1 h after removal of the filament, i.e. 2 h after induction of tMCAO.

**Brain infarct volume measurements**

The ischemic lesion was measured 24 hours after MCAO using TTC staining (6). The brain was cut in three (mice) or five (rats) 2mm thick coronal sections using a mouse/rat 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 Chemie GmbH, Munich, Germany. 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: $V_{\text{indirect}} (\text{mm}^3) = V_{\text{infarct}} \times (1-(V_{\text{ih}}-V_{\text{ch}}) / V_{\text{ch}})$, where the term $V_{\text{ih}}-V_{\text{ch}}$ 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. Brain edema volume can be calculated by subtracting corrected from uncorrected infarct volumes (7).

**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 (8) with the following definitions for mice and rats:
Mice: 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.

Rat: Score 0, no deficits; 1, flexion of the left forelimb; 2, flexion of the left forelimb and right side weakness; 3, occasional unidirectional circling behaviour; 4, occasional circling and longitudinal spinning; 5 no movement.

**Motor function**

Prior to sacrifice, the mice and rats were also scored for neurological motor deficits according to the Grip Test (9). 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, 50 cm 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).

**Protein Extraction and Western Blot Analysis**

Western blot analysis was performed according to standard procedures (10). The following primary antibodies were used: polyclonal antibody (pAb) anti-occludin (ab31721, Abcam), and mAb anti–b-actin (A5441, Sigma-Aldrich).

**Blood-brain barrier function**

To determine the permeability of the cerebral vasculature and brain edema, 2% Evans blue tracer (Sigma Aldrich, Germany) diluted in 0.9% NaCl was injected intravenously at reperfusion. Measurement of Evans Blue extravasation was performed as described in (1).
**Oxidative stress: DHE staining**

The presence of ROS was determined using dihydroethidium (Sigma, stock solution 2mM) 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) 2ng/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).

**Cell death measurement**

Apoptotic neurons were visualized by TUNEL analysis on cryopreserved brain sections as described in (11). The TUNEL in situ death detection kit TMR red (Roche, Switzerland) was used according to the manufacturer’s instructions. Afterwards, slices were washed and subsequently covered with AquaTec (Merck, Darmstadt, Germany). All sections were analyzed with a Nikon Eclipse 50i microscope equipped with the DS-U3 DS camera control unit and NIS-Elements software (Nikon, Tokyo, Japan). Digital images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

Necrotic Neurons were visualized by Fluoro-Jade C (#AG325, Millipore) staining on paraffin embedded brain sections. The Fluoro-Jade C staining was performed as suggested by the manufacturer. Afterwards, slices were washed and stained with DAPI (1:1000 in PBS, #D9542, Sigma-Aldrich) and subsequently covered with CYTOSEAL XYL (8312-4 Thermo Scientific. All pictures were made with a DMi8 microscope (Leica Microsystems, Wetzlar) equipped with a Hamamatsu Orca Flash 4.0 V2 Camera (Hamamatsu, Herrsching) and the LASX software (Leica...
Microsystems). For cell counting and picture processing ImageJ (NIH, version 1.51p) was used.

**Murine model of hindlimb ischemia**

The right femoral artery was permanently ligated. The mouse was placed on a heating pad (UNO temperature control unit, UNO Roestvaststaal BV) and body temperature was monitored using a rectal probe and maintained at 37°C using a feedback-controlled infrared lamp. A suture (5-0 silk) was placed 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. Animals were sacrificed 4 weeks after femoral artery ligation.

In the study, 23 WT and 17 KO animals were used. Two of the 25 WT animals died due to bleeding complications during the surgery. A Doppler measurement after the surgery was performed to exclude animals with no cessation of blood flow (**SI Appendix, Table S3**). This post-doppler measurement confirmed correct ligation of the artery and thus cessation of the blood flow in all of the animals.

**Doppler measurements**

Doppler measurements were done before and directly after surgery, at day 3, day 14 and day 28 after surgery. Mice were anesthetized and placed on the heating plate of the Moor Laser-Doppler (Moor LD12™, Moor Instruments Ltd Millwey Axminster Devon, UK). The mouse was allowed to heat up for 10 minutes before starting the scan. Three consecutive scans were made for each mouse. An area of interest was drawn around the paws and the mean color pixel value was calculated per paw and expressed as ratio of ligated over non-ligated leg.
**Capillary density**

Muscle samples were dissected and formalin-fixed. Paraffin embedded sections (4μm) of the musculus adductor and musculus gastrocnemius were used for CD31 staining. Antigen retrieval was achieved by heat induced epitope retrieval using 0.01 M Citrate buffer (pH 6.0). Slides were incubated at 4°C overnight with the primary antibody, monoclonal rat anti-mouse antibody to CD31 (PECAM-1) (Histonova-Dianova, Cat. no DIA310) diluted 1:50. As secondary antibody, biotin labelled rabbit anti-rat antibody (dakocytomotion Denmark no. E0468) was used diluted 1:200 (incubation for 30 minutes). Pictures were taken using a Leica camera connected to a Zeiss microscope. 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 square mm.

**Myocardial infarction and ischemia-reperfusion of the heart models**

Mice were anaesthetized with isoflurane in air (Abbott forene Isoflurane, 4-5% for induction, 2-3% for maintenance) and intubated per orally with a stainless-steel tube 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. Body temperature was monitored using a rectal probe and maintained at 37.0°C using a feedback-controlled infrared lamp and a heating plate. During surgery, an ECG was recorded with IDEEQ software (IDEE, Maastricht University). Using a left thoracotomy, the left descending coronary artery (LAD) was ligated with a 6-0 polyprolene suture permanently for myocardial infarction (MI). For the transient ischemia of 45 minutes, a small poly-ethylene tube was inserted under the ligature, compressing the coronary artery, which was then removed after the ischemic period. During the ischemic period, mice were kept under anesthesia. The chest was closed with 5-0 silk sutures (Ethicon).
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 in a thermoneutral temperature (28 °C). After 24 hours (short term transient ischemia), an ultrasound was performed followed by terminal hemodynamic characterisation (see below), then the heart was excised and used for infarct size measurements (see below TTC stain). At the end of the long-term experiment (28 days), hearts were quickly excised and the atria were removed. The ventricles were cut transversally at 3mm from the apex. The apical part was shock frozen for mRNA extraction. The basal part was fixed in formalin and processed for paraffin embedding.

**Animals included**

Short term study: In total, 46 KO and 48 WT animals were included in the complete study. For infarct size measurements, 19 WT and 18 KO animals were used. For one KO animal no reperfusion was seen, one WT animal did not have a visible ischemia and 3 WT animals died during surgery or before the endpoint of 24h. The remaining 17 WT and 16 KO animals were included in the infarct size analysis using the intention to treat principle ([SI Appendix, Table S3](#)). For ultrasounds, data were excluded if one of the repeated measurements was missing resulting in 39 WT and 36 KO animals. The hemodynamic measurements were performed in 11WT and 16KO animals.

Long term study: For the permanent ischemia, from the 34 WT and 30 KO animals included in the study, 8 and 5 animals respectively died due to cardiac rupture or heart failure before the end of the experiment. The remaining 26 WT and 25 KO animals were included in the infarct size analysis using the intention to treat principle. For the transient model, 24 WT and 24 KO animals were included in the study of which 8 WT and 6 KO animals died before the end of the experiment. One WT and 1 KO animal could not be included in the infarct size analysis due to technical problems. The
remaining 15WT and 17KO mice were included according to intention to treat principle (SI Appendix, Table S3). For the ultrasounds, data were excluded if one of the repeated measurements was missing resulting in 21 WT and 23 KO animals for the permanent and 14WT and 16KO for the transient model. The hemodynamic measurements were performed in 20WT and 22KO animals for the permanent and 11WT and 15KO for the transient model, the other animals died just before or during the measurement due to heart failure.

Echocardiography

In vivo echocardiography measurements were performed under light isoflurane anaesthesia before the surgery and at day 1 (short term) or 14 and 28 (long term), using the Vevo 2100 echocardiography system (Visualsonics, Toronto, Canada). Two-dimensional B-Mode echocardiograms were captured at a rate of 90-120 Hz from parasternal long-axis views as well as from mid-papillary short axis-views of the left ventricle. Data were obtained from at least 3 different images taken in end-diastole and systole using the accompanying software from the Vevo 2100 echocardiography system. From the long-axis echocardiograms, the ejection fraction (EF) was defined as 100* (EDV-ESV)/EDV) (12, 13).

Evaluation of left ventricular contractility

Left ventricular contractility was measured at day 1 or 28 before sacrifice. Mice were anaesthetized with urethane 2.5mg/kg intraperitoneally (Sigma). Body temperature and respiration were controlled as described above. A high-fidelity pressure transducer (Mikro-tip, 1.4F, SPR-671 Millar Instruments, Houston, TX, USA) was inserted into the left ventricle via the right carotid artery. Ventricular pressure was measured and sampled at a rate of 2kHz. After a baseline measurement, the heart was stimulated by intravenous infusion of increasing doses of dobutamine (Sigma) via
a microinjection pump (Model 200 series, KdScientific, Boston, MA, USA) starting at 90μg/min and increasing by 90μg every two minutes to a maximum dose of 540μg/min. Heart rate, maximal positive pressure and minimal positive pressure were calculated for every infusion rate using IdeeQ software.

**Evaluation of infarct size short term study**

After reopening the chest, the left descending coronary artery was ligated again at the same spot. Then, Evans Blue ink was injected via the inferior vena cava and allowed to spread through the vascular system. Then, the hearts were quickly excised and frozen. After freezing, the hearts were cut into 4-5 slices of 2mm and soaked in TTC (2% in PBS) for 30 minutes. Pictures were taken with a Dinolite camera connected to the microscope. Area at risk and infarct size were were analysed using the Leica Qwin pro v3.5.1 software. Infarct sizes are expressed as percentage of total left ventricular area.

**Evaluation of infarct size long term study**

Infarct sizes were calculated from paraffin-embedded left ventricular sections stained with AZAN. Sections of 4μm were incubated in preheated AZAN I solution for 30 minutes at 37°C, followed by rinsing in demineralized water and incubation in 5% phosphotungstic acid for 45 minutes. After rinsing in tap water, slides were finally incubated in AZAN II solution (diluted 1:3 in demineralized water) for 10 minutes. Pictures were taken using a Leica camera connected to a Zeiss microscope. 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.

**Hippocampal brain slices**

In vitro damage caused by oxygen-glucose deprivation/re-oxygenation and the protection elicited by VAS2870 and GKT136901 was studied in acutely isolated rat
hippocampal slices. Brains from 2-3 months old adult male Sprague-Dawley rats (250-300 g) or Nox4 KO mice (2-3 months) were isolated as described previously (14). Rats or mice were quickly decapitated and forebrains were rapidly removed from the skull and placed into ice-cold Krebs bicarbonate dissection buffer (pH 7.4), containing (in mM): NaCl 120, KCl 2, CaCl$_2$ 0.5, NaHCO$_3$ 26, MgSO$_4$ 10, KH$_2$PO$_4$ 1.18, glucose 11 and sucrose 200. The chamber solutions were pre-bubbled with either 95% O$_2$/5% CO$_2$ or 95% N$_2$/5% CO$_2$ gas mixtures, for at least 30 min before slice immersion, to ensure O$_2$ saturation or removal. The hippocampi were quickly dissected and 300mm thick slices were cut using a McIlwain Tissue Chopper. Then, the slices were transferred to vials containing a sucrose-free dissection buffer, bubbled with 95% O$_2$/5% CO$_2$ in a water bath at 37°C for 45 min, to allow tissue recovery (equilibration period). Oxygen and glucose deprivation was induced by incubating the slices for a 15 min period in a glucose-free Krebs solution (glucose was replaced by 2-deoxyglucose), equilibrated with a 95% N$_2$/5% CO$_2$ gas mixture. Slices incubated for 15 min in a modified Krebs solution (in mM: NaCl 120, KCl 2, CaCl$_2$ 2, NaHCO$_3$ 26, MgSO$_4$ 1.19, KH$_2$PO$_4$ 1.18 and glucose 11), equilibrated with 95% O$_2$/5% CO$_2$ served as controls. After the OGD period, the slices were returned back to an oxygenated normal Krebs solution containing glucose for 2h (re-oxygenation period), during which VAS2870 (10 𝜇𝑀) or GKT136901 (0.1 𝜇𝑀) were added as treatments do the rat studies. In case of Nox4 KO mice, no treatment was used.

**Cell viability of hippocampal slices**

Hippocampal cell viability was determined using the colorimetric MTT assay (14). Hippocampal slices were collected immediately after the re-oxygenation period and were incubated with MTT (0.5 mg/ml) in Krebs bicarbonate solution for 30 min at 37°C. The tetrazolium ring of MTT can be cleaved by active dehydrogenases in viable cells,
producing a precipitated formazan derivative. This formazan derivative was solubilized by adding 200 µl DMSO. The optical density was measured spectrophotometrically at 540 nm using a micro plate reader. Absorbance values obtained in control slices were set to 100% viability and experimental variables were normalized with respect to this value.

**ROS determination in hippocampal brain slices**

To measure cellular reactive oxygen species (ROS), we used the molecular probe \( \text{H}_2\text{DCFDA} \) (34). Immediately after chopper sectioning, 300µm thick hippocampal slices were loaded with \( \text{H}_2\text{DCFDA} \) (10 µl/ml) for 40 min in Krebs solution. Subsequently, the slices were washed once with Krebs solution during 10 min and OGD/Re-Ox protocol started. Fluorescence was measured in each slice using a fluorescence inverted NIKON eclipse T2000-U microscope (Izasa, Madrid, Spain). Wavelengths of excitation and emission of \( \text{H}_2\text{DCFDA} \) were 485 and 520 respectively. Images were taken at CA1 at magnifications of 100x. Fluorescence analysis was performed using the Metamorph programme version 7.0. Fluorescence under basal conditions was set to 1 and experimental variables were normalized with respect to this value.

**Human brain microvascular endothelial cells (HBMEC) culture subjected to hypoxia**

HBME cells (Cell systems, USA) between passage 3 and 9 were cultured to approximately 90% confluence using specialized cell medium (EGM-2 MV BulletKit, Lonza, The Netherlands) enriched with 5% fetal bovine serum FBS before starting the hypoxia period. For cell studies, HBMECs were seeded at specific density (6\( \times \)10^4 cells/ml) in 12 wells-plates and incubated during 24h at 37°C. Later, cell medium was replaced for non-FBS containing medium (2ml/well) following by 6h of hypoxia (94,8% \( \text{N}_2 \), 0.2% \( \text{O}_2 \) and 5% \( \text{CO}_2 \)) at 37°C using hypoxia workstations (Ruskin Invivo2 400
station, The Netherlands). The hypoxia period was followed by 24h of reperfusion in presence or absence of 1 µM GKT136901 (Genkyotech, Switzerland) as treatment. Control cells were exposed to normoxia (75% N₂, 20% O₂ and 5% CO₂) and enriched medium during the hypoxia period. All flasks and well plates were pre-treated with fibronectin (Sigma-Aldrich, The Netherlands) solution (1:100 in PBS).

**Assessment of cell viability in HBMEC**

After 24h reperfusion, cell viability was assessed using the colorimetric MTT assay (14). MTT solution (5 mg/ml) was added to each well (100 µl/ml) and incubated for 2h at 37°C. The formazan salt formed was solubilized by adding 350 µl DMSO. The optical density was measured spectrophotometrically at 540 nm using a micro plate reader. Absorbance values obtained in control cells were set to 100% viability.

**Cell permeability in HBMEC**

For the passive diffusion assay 2 x 10⁴ HBMEC were grown to confluence on membranes of Transwell inserts (collagen-coated Transwell Pore Polyester Membrane Insert; pore size = 3.0 µm (Greiner Bio One, Frickenhausen, Germany or Corning, The Netherlands). 24h before inducing 6h of ischemic conditions followed by 24h reperfusion period where cells were treated with 1µM GKT136901. Dextran tracer: The passive permeability was assessed with 3kDa dextran tracer (fluorescein conjugated) and 70 kDa dextran tracer (Texas red conjugated) (Invitrogen, Waltham, MA USA). To load the tracer onto the HBMEC layer, the solution was removed from the upper and lower chamber of the Transwell system. The lower chamber was filled with ACSF solution (ACSF; 120 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 22 mM NaHCO₃, 2 mM MgSO₄, 2 mM CaCl₂, and 20 mM glucose), the upper chambers with 25µg/ml tracer, diluted in ACSF solution. After 5 min incubation
at 37°C. The amount of diffused tracer in the lower chamber was measured with Tecan infinite M200PRO (TECAN, Männedorf, Switzerland).

**Evans Blue extravasation:** Cell permeability was assessed using the Evans Blue dye (Sigma-Aldrich, The Netherlands). To initiate the diffusion experiments, the medium was removed and cells were washed once with assay buffer. The same buffer (1.5 ml) was added to the abluminal side of the insert. Permeability buffer (0.5 ml) containing 4% bovine serum albumin (Sigma-Aldrich, The Netherlands) and 0.67 mg/ml Evans blue dye was loaded on the luminal side of the insert followed by 15 min incubation at 37°C. The concentration of Evans Blue in the abluminal chamber was measured by determining the absorbance of 150 µl buffer at 630 nm using a microplate reader.

**Statistics**

All mice and in vitro data are expressed as mean ± SEM. Rat in vivo experiments are expressed as mean ± SD. Using the GraphPad Prism 5.0 software package statistical differences between mean values were determined by Student’s two-tailed t test (mice) and Mann-Whitney test for rat experiments. For repeated measurements a two-way ANOVA was used. Statistical comparisons between groups were performed using one-way ANOVA. Data were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test and then analyzed by one-way analysis of variance (ANOVA) with posthoc Bonferroni adjustment for P values. The numbers of animals necessary to detect a standardized effect size on infarct volumes ≥ 0.2 were determined via a priori sample size calculation with the following assumptions: α = 0.05, power of 80%, a minimal assessable treatment effect of 40% and a variation of 20% (GraphPad Stat Mate 2.0; GraphPad Software). Nonparametric functional outcome scores were compared by Kruskal-Wallis test with posthoc Dunn multiple
comparison test. For comparison of survival curves the log-rank test was used. 
P values < 0.05 were considered statistically significant.
**Figure S1.** Induction of NOX4 expression in four different ischemic conditions: hindlimb ischemia, myocardial infarction, brain ischemia and a human brain microvascular endothelial cells (HBMEC) ischemia model. (A) Relative gene expression of Nox4 was upregulated in wild type mice subjected to a permanent ligation of the femoral artery in comparison with sham-operated mice (* P < 0.05, control n = 6, hindlimb n = 10). (B) Relative gene expression of Nox4 showed a 40 times increase in wild type mice subjected to occlusion of the left descending coronary artery compared to sham-operated mice (* P < 0.05, control n = 8, myocardial infarction n = 7). (C) Nox4 gene expression in human brain microvascular endothelial cells (HBMEC) subjected to 6h of hypoxia (0.2% O₂) was significantly increased in comparison with HBMEC subjected to normoxia conditions (** P < 0.01, n = 5). (D) Nox4 expression in brain tissue from mice subjected to a transient occlusion of the middle cerebral artery (1h) was significantly increased in comparison with non-stroked animals (* P < 0.05, stroke n = 4, non-stroke n = 4).
Figure S2. Role of NOX4 in ischemia-reperfusion of the heart on long term (A/B/C) and short term (D/E/F) effects. (A) No significant differences in infarct size 4 weeks after ischemia-reperfusion between Nox4 KO (red, n = 17) and WT mice (black, n = 15). (B) Ejection fraction decreased after heart ischemia-reperfusion showed no significant change between Nox4 KO (red, n = 16) and WT mice (black, n = 14). (C) Left ventricular function was not different between NOX4 KO (red, n=15) and WT mice (black, n =11). (D) No significant differences in infarct size 24h after ischemia-reperfusion between Nox4 KO (red, n = 16) and WT mice (black, n = 17). (E) Ejection fraction decreased after heart ischemia-reperfusion with no significant change between Nox4 KO (red, n = 36) and WT mice (black, n = 39). (F) Left ventricular
function was not different between Nox4 KO (red, n = 16) and WT mice (black, n = 11). Representative staining pictures are shown above each graph.
Figure S3. Generation of cell-specific Nox4 KO mice. For endothelial-cell specific NOX4 KO mice (eKO, dark blue), female mice homozygous for the floxed NOX4 gene were bred with normal C57/Bl6 male mice that express the Cre recombinase gene under control of the endothelial-cell specific Tie2 promotor. Smooth muscle cell-specific (sKO, intermediate blue) and neuron specific Nox4 KO (nKO, light blue) mice were generated in an analogous way using SMMHC-Cre+ mice and CaMKII-Cre+ mice respectively. Deletion of Nox4 in SMC and neurons was Tamoxifen-inducible. sWT and nWT mice were also injected with Tamoxifen for proper comparison.
Figure S4. Contribution of endothelial and neuronal NOX4 in necrosis. (A) Contralateral side: No difference in number of cortical necrotic neurons was found in endothelial Nox4 KO (eKO) and neuronal Nox4KO (nKO) mice in comparison with WT animals. (B) Ipsilateral side: nKO mice presented less necrotic cells in comparison with nWT (** P < 0.01, n = 8) while no effect was shown in eKO mice (n = 8).
Figure S5. Protein expression of beclin-1 in brain tissue from endothelial NOX4 KO (eKO) and WT mice post-stroke. Significant reduction of beclin-1 protein expression after brain ischemia (tMCAO) has been found in eKO mice compared to their respective WTs mice (* P < 0.05, n = 6).
Figure S6. Nox4 KO rat generation. (A) Microinjections of Zinc Finger Nucleases (ZFN) pairs targeting exon 14 and 15 into wildtype female WKY rats resulted in the respective loss of exons 14 and 15. Specific NOX4 ZFN-binding sequence (red) and ZFN cleavage site (blue). (B) Representation of the Nox4 KO deletion product. Flanking primers for the deletion regions, forward and reverse are represented in red (F and R).
Figure S7. NOX4 KO rat genotyping. Control and Nox4 KO rat tail genomic DNA were purified and a PCR was performed to amplify the sequence covering exons 14 and 15 of the Nox4 gene (See material and methods for details). WT animals showed the complete DNA fragment (383bp) while in KO rats the mutated gene was detected (303bp). Both WT and KO bands were shown in heterozygote animals.
Figure S8. Cell death and ROS formation are significantly reduced in hippocampal brain slices from Nox4 KO mice. (A) Cell death was significantly reduced in hippocampal brain slices from global Nox4 KO mice compared to its respective WT littermates (* P < 0.05, n = 4). (B) ROS formation was also decreased in hippocampal brain slices subjected to OGD in comparison with WT OGD-treated slices (** P < 0.01, *** P < 0.001, n = 4).
Figure S9. GKT136901 increases cell viability in human brain microvascular endothelial cells (HBMEC) subjected to OGD/Re-oxygenation (Re-Ox). (A) Experimental protocol. To promote cell seeding, HBMEC were incubated during 24h under physiological conditions followed by 6h of hypoxia period and 24h of Re-Ox. 1 µM GKT136901 was added at the beginning of the Re-Ox period (time=0 after hypoxia). (B) Cell viability was significantly increased in cells treated with 1 µM GKT136901 (** P < 0.01, n = 6) in comparison with non-treated cells (### P < 0.001, n = 6).
Figure 10. Effects of GKT136901 treatment on dextran tracer and Evans Blue (EB) permeability in human brain microvascular endothelial cells (HBMEC) subjected to hypoxia/Re-oxygenation (Re-Ox). (A) Passive permeability was assessed with a 3 kDa dextran tracer (fluorescein conjugated) and (B) a 70 kDa dextran tracer after 6h of hypoxia followed by 24h of re-oxygenation period in presence or absence of 1 µM GKT136901. Cell permeability was significantly increased after OGD (## P < 0.01, # P < 0.05, n = 5) while NOX4 inhibition post-OGD prevented this detrimental effect (´ P < 0.05, n = 5). (C) Cell permeability was also assessed by measuring EB fluorescence post-OGD. EB diffusion was significantly reduced in treated cells (1 µM GKT136901) (## P < 0.05, n = 3) in comparison with non-treated cells (´ P < 0.05, n = 3).
Fig S11. GKT136901 treatment after 1h occlusion of the middle cerebral artery (tMCAO) in Nox4 KO mice. Infarct volume was significantly reduced in Nox4 KO (no treatment) and Nox4 KO animals treated with GKT136901 (10 mg/Kg) in comparison with WT mice (** P < 0.01, n = 5). No difference was detected when comparing Nox4 KO (n = 3) and Nox4 KO mice (n = 3) treated with GKT136901 (10 mg/Kg) after 1h tMCAO. Complete sets of brain slices from a representative animal (TTC staining) are shown above the graph.
## Supplementary tables

### Table S1. NOX4 gene expression in bone marrow of WT and Tie2-Cre mice

<table>
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<th>Animal number</th>
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<th>NOX2 expression (Ct)</th>
<th>β-actin (Ct)</th>
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NOX, NADPH oxidase, WT, wild type.
Table S2. mRNA levels of NOX1, NOX2 and NOX4 in brain, kidney and lung samples from NOX4 KO/WT rats

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<td>ΔCt</td>
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<td>WT Sample Ct</td>
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<td>ΔCt</td>
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<td>ΔCt</td>
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<td>WT - Lung</td>
<td>20.81</td>
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NOX, NADPH oxidase, WT, wild type; KO, Knock-out.
Table S3. Animals excluded from the statistical analysis after tMCAO, myocardial infarction and hindlimb ischemia

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NOX4, NADPH oxidase 4; WT, wild type; KO, Knock-out; sNOX4, smooth muscle cells NOX4 KO; nNOX4, neuronal NOX4 KO; eNOX4 KO, endothelial NOX4 KO; tMCAO, transient occlusion of the middle cerebral artery; TTC, 2,3,5- triphenyltetrazolium hydrochloride.

Animal exclusion procedures are described in the respective methods parts.
References


