



# Deletion of the ageing gene p66<sup>Shc</sup> reduces early stroke size following ischaemia/reperfusion brain injury

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## Aims

Stroke is a leading cause of morbidity and mortality, and its incidence increases with age. Both in animals and in humans, oxidative stress appears to play an important role in ischaemic stroke, with or without reperfusion. The adaptor protein p66<sup>Shc</sup> is a key regulator of reactive oxygen species (ROS) production and a mediator of ischaemia/reperfusion damage in *ex vivo* hearts. Hence, we hypothesized that p66<sup>Shc</sup> may be involved in ischaemia/reperfusion brain damage. To this end, we investigated whether genetic deletion of p66<sup>Shc</sup> protects from ischaemia/reperfusion brain injury.

## Methods and results

Transient middle cerebral artery occlusion (MCAO) was performed to induce ischaemia/reperfusion brain injury in wild-type (Wt) and p66<sup>Shc</sup> knockout mice (p66<sup>Shc</sup><sup>-/-</sup>), followed by 24 h of reperfusion. Cerebral blood flow and blood pressure measurements revealed comparable haemodynamics in both experimental groups. Neuronal nuclear antigen immunohistochemical staining showed a significantly reduced stroke size in p66<sup>Shc</sup><sup>-/-</sup> when compared with Wt mice ( $P < 0.05$ ,  $n = 7-8$ ). In line with this, p66<sup>Shc</sup><sup>-/-</sup> mice exhibited a less impaired neurological function and a decreased production of free radicals locally and systemically ( $P < 0.05$ ,  $n = 4-5$ ). Following MCAO, protein levels of gp91phox nicotinamide adenine dinucleotide phosphate oxidase subunit were increased in brain homogenates of Wt ( $P < 0.05$ ,  $n = 4$ ), but not of p66<sup>Shc</sup><sup>-/-</sup> mice. Further, reperfusion injury in Wt mice induced p66<sup>Shc</sup> protein in the basilar and middle cerebral artery, but not in brain tissue, suggesting a predominant involvement of vascular p66<sup>Shc</sup>.

## Conclusion

In the present study, we show that the deletion of the ageing gene p66<sup>Shc</sup> protects mice from ischaemia/reperfusion brain injury through a blunted production of free radicals. The ROS mediator p66<sup>Shc</sup> may represent a novel therapeutic target for the treatment of ischaemic stroke.

## Keywords

Stroke • Ischaemia • p66Shc • ROS

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## Introduction

Stroke is a leading global cause of mortality, responsible for more deaths than cancer.<sup>1</sup> Even in patients presenting comparable degrees of ischaemia, a huge variability in the recovery of brain function is observed, indicating a complex pathological process which, to date, still lacks a specific therapy for its effective treatment.<sup>2</sup> Indeed, even reopening of the stroke-related artery with either thrombolysis or catheter intervention is still far from providing a safe and effective therapy for the majority of patients. It thus appears crucial to elucidate the molecular mechanisms underlying the pathogenesis of neuronal injury after stroke to set the basis for the design of novel effective therapeutical strategies.

Reactive oxygen species (ROS) are considered as crucial players in cerebrovascular disease.<sup>3,4</sup> Several animal and human studies showed an association between ischaemic stroke and increased systemic and local production of ROS.<sup>5–9</sup> A large body of evidence indicates nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) as a major source of ROS generation in cerebrovascular disease.<sup>3,10</sup> In line with this, NADPH oxidase expression is profoundly greater in the vessel wall of cerebral arteries rather than systemic arteries<sup>11</sup> and its genetic deletion in mice reduces brain infarction.<sup>12</sup> Recently, NADPH oxidase has been shown to be a downstream target of the adaptor protein p66<sup>Shc</sup>.<sup>13</sup>

The mammalian p66<sup>Shc</sup> adaptor protein, together with p46<sup>Shc</sup> and p52<sup>Shc</sup>, belongs to ShcA adaptor/docking protein family, which plays an important role in transducing activation signals from receptors, such as growth factor, cytokines, and integrins, to downstream signalling cascades.<sup>14,15</sup> Among the three isoforms, p46<sup>Shc</sup> and p52<sup>Shc</sup> are important in the regulation of growth factor-induced Ras/Erk signalling,<sup>14,15</sup> whereas p66<sup>Shc</sup> is crucially involved in ROS generation and translates oxidative stress into apoptosis.<sup>14</sup> Genetic deletion of p66<sup>Shc</sup> in mice extends lifespan by 30%<sup>16</sup> and slows down the progression of atherogenesis in double-mutant p66<sup>Shc</sup><sup>-/-</sup>/ApoE<sup>-/-</sup> mice fed on a high fat diet.<sup>17,18</sup> Deletion of p66<sup>Shc</sup> also protects from hyperglycaemia-induced endothelial dysfunction,<sup>19,20</sup> reduces fat accumulation and premature death in adipose tissue,<sup>21,22</sup> and attenuates glomerulopathy<sup>23,24</sup> in diabetic mice. The reported protective effects achieved by p66<sup>Shc</sup> deletion are mainly due to reduced oxidative stress, improved insulin sensitization, increased mitochondrial uncoupling, and reduced triglyceride accumulation.<sup>17–19,21–27</sup> In line with this, increased levels of p66<sup>Shc</sup> mRNA have been reported in peripheral blood mononuclear cells of type-2 diabetic patients<sup>25</sup> and in patients with acute myocardial infarction.<sup>28</sup> Enhanced expression of p66<sup>Shc</sup> has been reported in ethanol-induced liver damage in mice,<sup>29</sup> chronic kidney dysfunction in spontaneous hypertension rats<sup>30</sup> and aged rats,<sup>30</sup> and HIV-1-induced cell apoptosis in podocyte,<sup>31</sup> which is associated with a lower level of ROS production and a blunted activation of NfκB.<sup>29,30</sup> Of note, deletion of p66<sup>Shc</sup> was also shown to protect *ex vivo*-perfused murine hearts from ischaemia/reperfusion-induced injury<sup>32</sup>; however, the involvement of p66<sup>Shc</sup> in stroke is largely unknown.

In the present study, we thus analysed the effects of genetic deletion of p66<sup>Shc</sup> in ischaemia/reperfusion brain injury. In particular, we investigated whether p66<sup>Shc</sup> knockout mice are protected from

ischaemia/reperfusion-induced and ROS-mediated brain injury and neurological deficits.

## Methods

### Animal model

Experiments were performed on 12–14-week-old wild-type (Wt) (C57Bl6j) and p66<sup>Shc</sup> male knockout mice (p66<sup>Shc</sup><sup>-/-</sup>). Animals were fed on a normal chow diet and had *ad libitum* access to food and water and were maintained at 24°C under a 12 h light/dark cycle. Study design and experimental protocols were approved by the institutional animal care committee (Licence no. TVA 139\_2008; Kommission für Tierversuche des Kantons Zürich, Switzerland).

### Middle cerebral artery occlusion model and haemodynamics measurements

To induce ischaemia/reperfusion brain injury, a transient middle cerebral artery occlusion (MCAO) surgery was performed on both p66<sup>Shc</sup><sup>-/-</sup> and Wt mice as previously described.<sup>33</sup> Mice were initially anaesthetized with 4% of isoflurane and then maintained on 1.5% isoflurane vaporized in NO<sub>2</sub> and O<sub>2</sub> (2:1). Following a midline cervical incision, the left common carotid artery (CCA), external artery (ECA), and internal carotid artery (ICA) were carefully exposed under an operating microscope. Thereafter, a 6-0 silicone-coated filament (Doccol Corporation, Redlands, CA, USA) was introduced into the CCA and advanced into the ICA ~9–12 mm from the common carotid bifurcation. Rectal temperature was maintained at 37 ± 0.5°C while the animals were under anaesthesia through the use of circulating water pads. The thread was left in place for 60 min. After the removal of the thread and wound care, animals were carefully observed and cared for and left in their cages for the next 24 h. The same procedure was performed for sham-operated animals. However, the silicone-coated filament was advanced into the ICA ~5 mm from the common carotid bifurcation, without interruption of cerebral blood flow (CBF) in the middle cerebral artery. During anaesthesia, regional CBF (rCBF) in the area of the cortex supplied by the MCA was measured using laser Doppler flowmetry (PeriFluxSystem 5000 with probe model no. 418-1, Perimed AB, Järfälla, Sweden). The microtip probe was positioned and glued ~2 mm posterior and 6 mm lateral to the bregma.

For blood pressure measurements, mice were allowed to familiarize with the procedure and equipment for a period of 1 week and then systolic (SBP) and diastolic blood pressure (DBP) and heart rate were recorded using the tail-cuff method (model LE 5002, Storage Pressure Meter, Letica, Spain).

MCAO experiments were performed blindly.

### Neurological deficit measurement

Neurological deficit measurement was performed after 1 and 24 h of reperfusion following MCAO, using a four-point scale based on the Bederson test<sup>34</sup>: normal motor function was scored as 0, flexion of the contralateral torso and forelimb on lifting the animal by the tail as 1, circling to the contralateral side but normal posture at rest as 2, leaning to the contralateral side at rest as 3, and no spontaneous motor activity as 4. Neurological deficit measurements were performed by two people independently in a blinded way.

### Stroke size measurement

Following 24 h of reperfusion, mice were sacrificed and perfused with PBS and relevant organs were excised. Brains were immersed in 4%

formalin in phosphate buffer overnight and transferred to 30% sucrose solution for at least 3 days. Next, coronal 30  $\mu\text{m}$ -thick sections were cut on a freezing microtome (Leica, Nussloch, Germany). Stroke size was measured by immunohistochemical neuronal nuclear antigen (NeuN) as previously performed (Dilution 1:100, Millipore, Temecula, CA, USA).<sup>35</sup> Quantification of stroke size was performed using the NIH ImageJ software.

## Western blotting

Protein expression was determined by western blot analysis. Isolated brains, basilar arteries, and middle cerebral arteries were homogenized in lysis buffer (NaCl 150 mmol/L, EDTA 1 mmol/L, NaF 1 mmol/L, DTT 1 mmol/L, aprotinin 10  $\mu\text{g}/\mu\text{L}$ , leupeptin 10  $\mu\text{g}/\mu\text{L}$ ,  $\text{Na}_3\text{VO}_4$  0.1 mmol/L, PMSF 1 mmol/L, and NP-40 0.5%). Protein concentration was measured according to the manufacturer's recommendations (Bio-Rad Laboratories GmbH, München, Germany). Equal amounts of protein were separated on a 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore, Volketswil, Switzerland) by semi-dry transfer. Antibodies against gp91phox, p67phox, and p47phox (all from Upstate) were used at 1:500 dilution. Anti-Shc (Cell Signaling, Danvers, MA, USA) was used at 1:1000 dilution. Blots were normalized to  $\alpha$ -tubulin (1:20 000 dilution, Sigma) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (1:20 000 dilution, Millipore, Temecula, CA, USA). Anti-rabbit and anti-mouse secondary antibodies were purchased from GE Healthcare (Buckinghamshire, UK). For the detection of protein carbonylation, dinitrophenol antibody (LifeSpan BioSciences, Inc.) at 1:200 dilution was used. All western blots were quantified by densitometric analysis performed using the Scion Image Corporation software.

## Measurement of reactive oxygen species

$\text{O}_2^-$  production in whole blood was determined using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (Noxygen, Germany). Blood samples were mixed with Krebs-HEPES solution containing sodium diethyldithiocarbamate trihydrate (5  $\mu\text{mol/L}$ ), deferoxamine methanesulfonate (25  $\mu\text{mol/L}$ ), and 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (200  $\mu\text{mol/L}$ ) (all from Noxygen, Germany), and heparin (100 U/mL). Samples were frozen in liquid nitrogen, and  $\text{O}_2^-$  production was assessed with an ESR spectrometer (Bruker, Bremen, Germany) with the following instrumental settings: centre field: 3485 G; sweep width: 50.000 G; static field: 3477 G; microwave frequency: 9.76 GHz; microwave power: 19.91 mW; modulation amplitude: 2.60 G; modulation frequency: 86.00 kHz; sweep time: 5.24 s; number of scans: 10.

## Statistical analysis

Data are given as mean  $\pm$  SEM. Statistical analysis was done by two-way ANOVA analysis of variance with *post hoc* multiple comparisons using the Bonferroni test, or paired/unpaired *t*-test in a two-tailed way as appropriate. A probability value  $P < 0.05$  denoted a significant difference. Statistical analyses were performed using the GraphPad Prism software version 4.03 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### Reduced brain infarction after ischaemia/reperfusion injury in $\text{p66}^{\text{Shc}/-}$ mice

To evaluate the role of  $\text{p66}^{\text{Shc}}$  in ischaemia/reperfusion brain injury, a transient MCAO surgery was performed on both  $\text{p66}^{\text{Shc}/-}$  and Wt mice in order to induce a focal ischaemic stroke. Middle

cerebral artery occlusion is a reliable and reproducible rodent model of cerebral ischaemia in humans that has been demonstrated to result in sensorimotor and cognitive deficits.<sup>33</sup> Following 60 min of MCA occlusion, the silicone filament was withdrawn, allowing reperfusion for 24 h. Following 24 h of reperfusion, both  $\text{p66}^{\text{Shc}/-}$  ( $P < 0.0001$ ) and Wt ( $P = 0.0086$ ) mice underwent a comparable weight loss (data not shown). To quantify stroke size, NeuN immunohistochemical staining was performed as previously described.<sup>35</sup> Morphometrical analysis revealed a significantly reduced stroke size in  $\text{p66}^{\text{Shc}/-}$  mice compared with Wt mice (Wt:  $42.84 \pm 6.05 \text{ mm}^2$  vs.  $\text{p66}^{\text{Shc}/-}$ :  $16.66 \pm 7.95 \text{ mm}^2$ ;  $*P = 0.0196$ ;  $n = 7-8$ ; Figure 1A). Sham-operated Wt and  $\text{p66}^{\text{Shc}/-}$  did not display any lesions (data not shown).

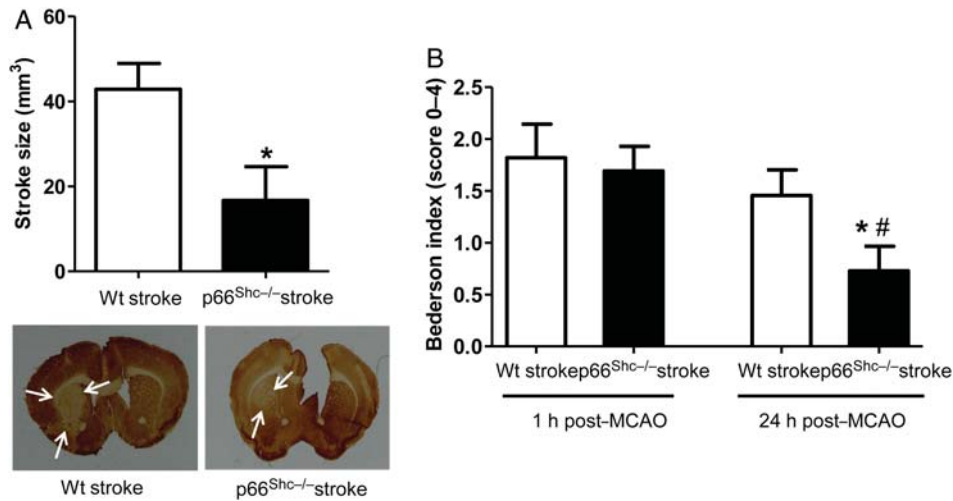
### Genetic deletion of $\text{p66}^{\text{Shc}}$ improves neurological function following middle cerebral artery occlusion

For the assessment of neurological function after MCAO, we performed a Bederson-based four-point scale test. After 1 h of reperfusion, both Wt and  $\text{p66}^{\text{Shc}/-}$  mice exhibited marked coordination dysfunction, shown as decreased activity, imbalance of movement, and decreased gripping ability (Wt:  $1.87 \pm 0.26$  vs.  $\text{p66}^{\text{Shc}/-}$ :  $1.43 \pm 0.17$ ;  $P = \text{NS}$ ;  $n = 14$ ; Figure 1B). Following 24 h of reperfusion,  $\text{p66}^{\text{Shc}/-}$  mice showed an improved neurological function compared with Wt mice (Wt:  $1.53 \pm 0.19$  vs.  $\text{p66}^{\text{Shc}/-}$ :  $0.89 \pm 0.33$ ;  $*P = 0.0142$ ;  $n = 14$ ; Figure 1B). Moreover, neurological deficit score of  $\text{p66}^{\text{Shc}/-}$  was improved at 24 h compared with 1 h of reperfusion (24 h:  $0.89 \pm 0.33$  vs. 1 h:  $1.43 \pm 0.17$ ;  $\#P = 0.0130$ ;  $n = 14$ ; Figure 1B). Such improvement was not observed in Wt mice (24 h:  $1.53 \pm 0.19$  vs. 1 h:  $1.87 \pm 0.26$ ;  $P = 0.2449$ ;  $n = 14$ ; Figure 1B), suggesting an improved recovery potential in  $\text{p66}^{\text{Shc}/-}$  mice. Both Wt and  $\text{p66}^{\text{Shc}/-}$  sham-operated mice did not exhibit any neurological deficit at 1 or 24 h (data not shown).

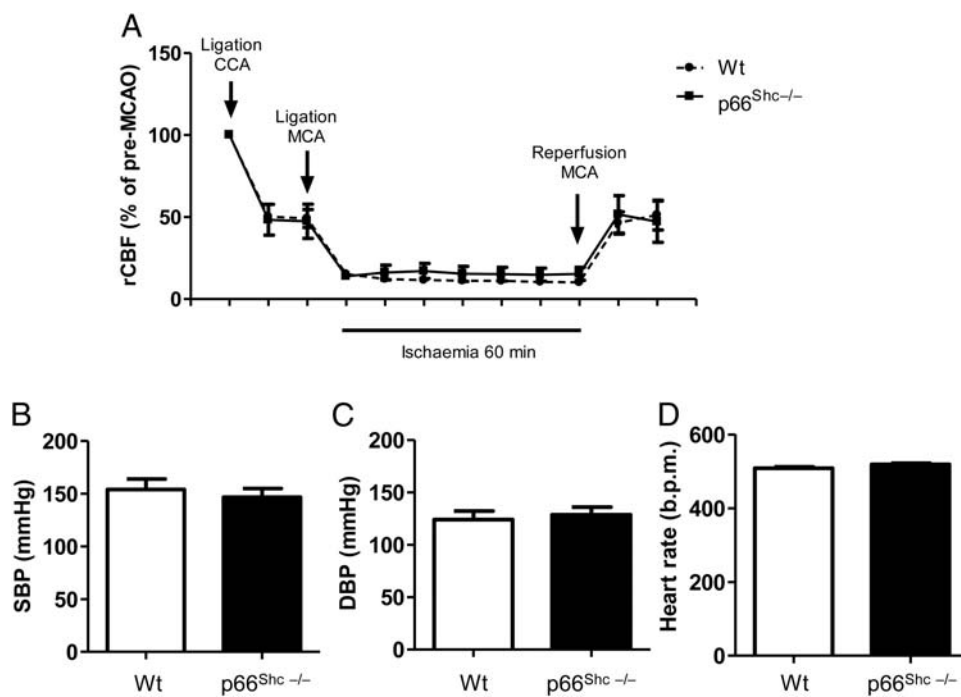
### Comparable cerebral perfusion and systemic blood pressure in wild-type and $\text{p66}^{\text{Shc}/-}$ mice

Regional cerebral blood flow was recorded during MCAO procedure using laser Doppler flowmetry. Basal rCBF was comparable between Wt and  $\text{p66}^{\text{Shc}/-}$  mice. Following the ligation of the CCA, a comparable reduction in blood flow was observed in both experimental groups (Wt:  $-49.6 \pm 2.69\%$  vs.  $\text{p66}^{\text{Shc}/-}$ :  $-51.67 \pm 9.37\%$ ; Figure 2A); similarly, after the insertion of the silicon thread to achieve MCAO, a comparable degree of rCBF reduction compared with basal level was recorded in Wt and  $\text{p66}^{\text{Shc}/-}$  mice, indicating a comparable degree of MCA occlusion in both groups (Wt:  $-84.86 \pm 2.49\%$  vs.  $\text{p66}^{\text{Shc}/-}$ :  $-86.13 \pm 2.77\%$ ; Figure 2A). Finally, upon the retraction of the silicone thread, a comparable re-establishment of blood flow was observed in both Wt and  $\text{p66}^{\text{Shc}/-}$  mice (Wt:  $53.58 \pm 6.82\%$  vs.  $\text{p66}^{\text{Shc}/-}$ :  $48.41 \pm 11.41\%$ ;  $P = \text{NS}$  for all time points;  $n = 6-7$ ; Figure 2A).

In order to exclude possible interference on stroke size by different systemic blood pressure in the two strains, blood pressure and heart rate were measured. No differences in SBP and DBP as well as in heart rate were observed in both experimental groups



**Figure 1** Stroke size and neurological deficit measurement after ischaemia/reperfusion brain injury. (A) Representative neuronal nuclear antigen immunohistochemical staining 24 h after middle cerebral artery occlusion (MCAO) in wild-type (Wt) and p66<sup>Shc</sup><sup>-/-</sup> mice. Arrows indicate subcortical and cortical infarct components. p66<sup>Shc</sup><sup>-/-</sup> mice show reduced stroke size compared with wild-type mice, \**P* = 0.0196. Values are given as cubic millimetre. (B) The Bederson test. Neurological deficit was measured either at 1 h of reperfusion or at 24 h of reperfusion. p66<sup>Shc</sup><sup>-/-</sup> stroke mice show improved neurological function at 24 h of reperfusion compared with wild-type stroke mice and with p66<sup>Shc</sup><sup>-/-</sup> stroke mice at 1 h of reperfusion. \**P* = 0.0142 for wild-type stroke (24 h) vs. p66<sup>Shc</sup><sup>-/-</sup> stroke mice (24 h). #*P* = 0.0130 for p66<sup>Shc</sup><sup>-/-</sup> stroke (24 h) vs. p66<sup>Shc</sup><sup>-/-</sup> stroke (1 h).



**Figure 2** Cerebral blood flow and arterial blood pressure measurements. (A) Laser Doppler measurements revealed no difference in regional cerebral blood flow before middle cerebral artery occlusion (MCAO) between wild-type and p66<sup>Shc</sup><sup>-/-</sup> mice and showed a similar alteration upon middle cerebral artery occlusion and reperfusion. (B–D) Wild-type (Wt) and p66<sup>Shc</sup><sup>-/-</sup> mice do not differ in systolic and diastolic blood pressure (SBP, DBP) as well as in heart rate.

(SBP: Wt:  $154 \pm 9.866$  mmHg vs.  $p66^{Shc-/-}$ :  $146.7 \pm 8.212$  mmHg;  $P = NS$ ; Figure 2B; DBP: Wt:  $124 \pm 8.083$  mmHg vs.  $p66^{Shc-/-}$ :  $128.7 \pm 7.219$  mmHg;  $P = NS$ ; Figure 2C; heart rate: Wt:  $508.3 \pm 3.930$  b.p.m. vs.  $p66^{Shc-/-}$ :  $518.7 \pm 3.283$  b.p.m.;  $P = NS$ ; Figure 2D).

### Ischaemia/reperfusion injury-induced local and systemic oxidative stress is reduced in $p66^{Shc-/-}$ mice

To address the role of oxidative stress, its level was quantified in whole-brain homogenates after ischaemia/reperfusion by measuring total protein carbonylation. Protein carbonylation is a widely accepted index of oxidative stress and the most common post-translational protein modification induced by oxidative stress. Ischaemia/reperfusion injury induced a significant increase in protein carbonylation in the brain of Wt stroke mice compared with that of sham-operated Wt mice (Wt stroke:  $207.1 \pm 40.3\%$  vs. Wt sham: 100%;  $*P < 0.05$ ;  $n = 4$ ; Figure 3A). In contrast, no change in brain oxidative stress levels after ischaemia/reperfusion was observed in  $p66^{Shc-/-}$  stroke mice compared with shams ( $p66^{Shc-/-}$  stroke:  $99.62 \pm 14.48\%$  vs.  $p66^{Shc-/-}$  sham:  $72.77 \pm 18.17\%$ ;  $P = NS$ ;  $n = 3-5$ ; Figure 3A).

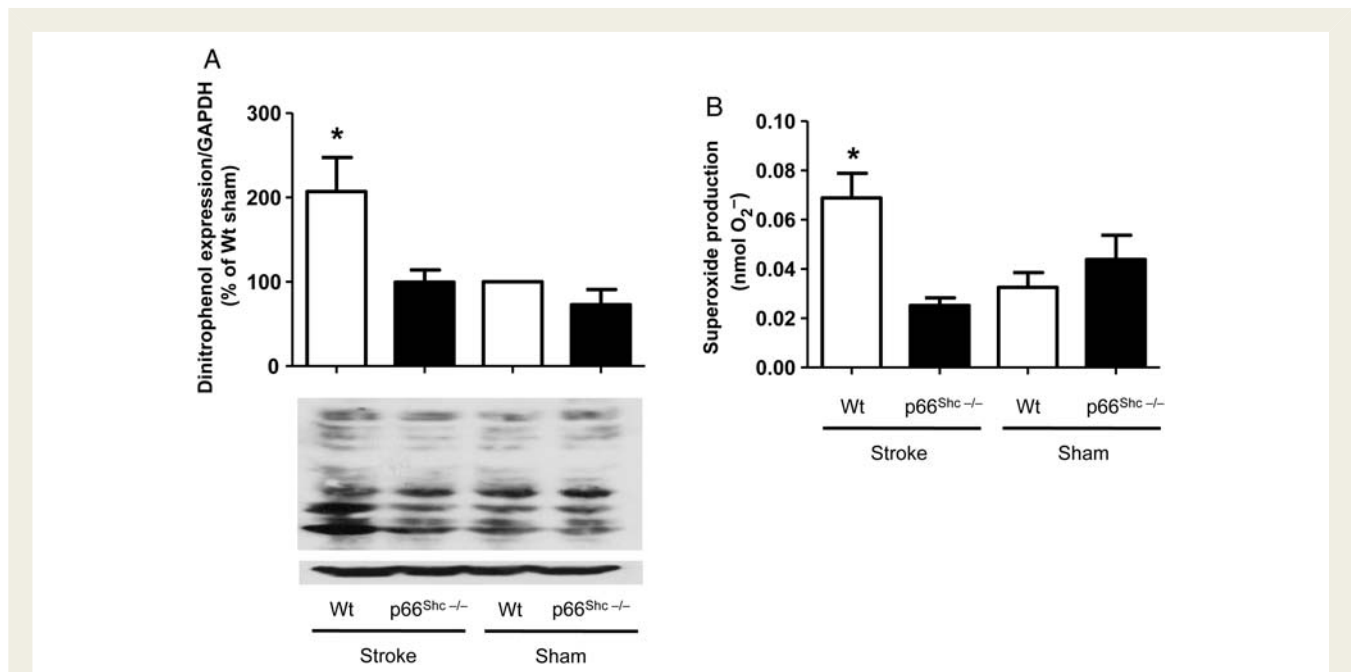
To determine levels of systemic oxidative stress following ischaemia/reperfusion injury,  $O_2^-$  levels were measured in whole blood using electron spin resonance spectroscopy. Wild-type stroke mice showed increased ROS generation after 24 h of reperfusion compared with Wt sham mice ( $0.068 \pm 0.009$  vs.  $0.032 \pm 0.005$  nmol;  $*P < 0.01$ ;  $n = 4-6$ ; Figure 3B). In contrast,

$p66^{Shc-/-}$  stroke mice displayed comparable levels of ROS to  $p66^{Shc-/-}$  sham mice ( $0.025 \pm 0.003$  vs.  $0.043 \pm 0.009$  nmol;  $P = NS$ ;  $n = 3-5$ ; Figure 3B).

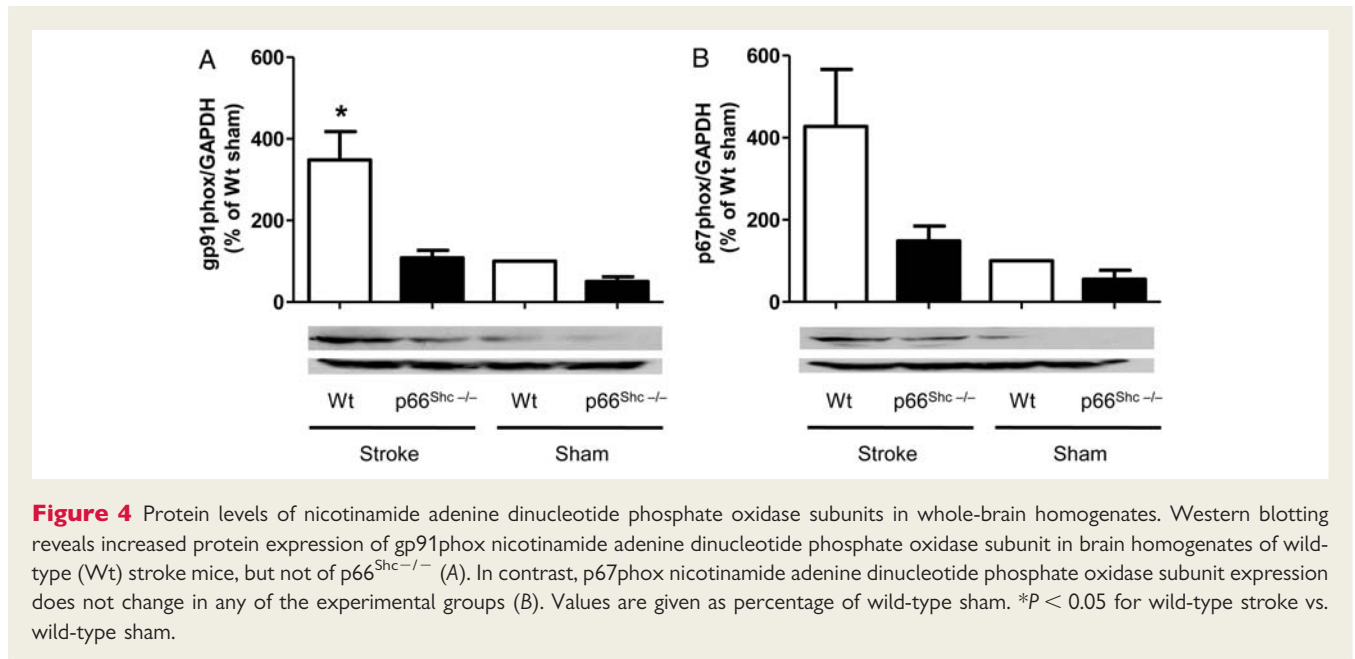
### Increased protein levels of gp91phox nicotinamide adenine dinucleotide phosphate oxidase subunit in wild-type mice after middle cerebral artery occlusion

Nicotinamide adenine dinucleotide phosphate oxidase is a well-known major source for ROS production in cerebrovascular disease as well as a downstream target of  $p66^{Shc}$ ; to this end, its expression was measured in brain homogenates 24 h after MCAO. In the present study, protein expression of gp91phox NADPH oxidase subunits, but not of p67phox and p47phox, was significantly increased in the brain of Wt stroke mice compared with that of Wt sham mice (gp91phox: Wt stroke:  $348 \pm 69.49\%$  vs. Wt sham: 100%;  $*P < 0.05$ ;  $n = 4$ ; Figure 4A). Interestingly, this increase was not observed in the brain of  $p66^{Shc-/-}$  stroke mice compared with that of  $p66^{Shc-/-}$  sham ones (gp91phox:  $p66^{Shc-/-}$  stroke:  $108.5 \pm 18.09\%$  vs.  $p66^{Shc-/-}$  sham:  $50.14 \pm 11.73\%$ ;  $P = NS$ ;  $n = 3-5$ ; Figure 4A). In contrast, p67phox (p67phox: Wt stroke:  $427.1 \pm 139.1\%$  vs. Wt sham: 100%;  $P = NS$ ;  $n = 3-5$ ; Figure 4B) and p47phox (data not shown) NADPH oxidase subunit protein expression did not change in any of the experimental groups.

To exclude possible involvement of other pro-/anti-oxidant enzymes, we looked at protein expression of COX-2, SOD1,



**Figure 3** Production of free radicals locally (brain) and systemically (in whole blood). (A) Wild-type (Wt) mice, but not  $p66^{Shc-/-}$  mice, show increased brain protein carbonylation after ischaemia/reperfusion brain injury. Values are given as percentage of wild-type sham-operated mice.  $*P < 0.05$  for wild-type stroke vs. wild-type sham. (B) Electron spin resonance spectroscopy measured increased  $O_2^-$  levels in whole blood of wild-type stroke mice but not in  $p66^{Shc-/-}$ . Values are given as nmol  $O_2^-$ .  $*P < 0.05$  for wild-type stroke vs. wild-type sham.



SOD2, and Gpx and found no significant differences in the levels of these proteins in Wt stroke vs. p66<sup>Shc-/-</sup> stroke groups (data not shown).

### Following ischaemia/reperfusion, p66<sup>Shc</sup> protein is increased in the basilar artery and middle cerebral artery but not in the whole brain

To determine the regulation of p66<sup>Shc</sup> expression in cerebral tissues following ischaemia/reperfusion, western blotting analysis was performed. p66<sup>Shc</sup> protein expression in the basilar artery of Wt stroke mice was profoundly increased compared with Wt sham mice (Wt stroke:  $615.4 \pm 257.1\%$  vs. Wt sham:  $100 \pm 15.26\%$ ; \**P* = 0.0245; *n* = 7–8; Figure 5A). In line with this, p66<sup>Shc</sup> protein levels in the middle cerebral artery of Wt stroke mice were also elevated compared with Wt sham mice (Figure 5B). In sharp contrast, hardly any p66<sup>Shc</sup> expression was detected in whole-brain homogenates and most importantly its levels remained unchanged after ischaemia/reperfusion (Figure 5C).

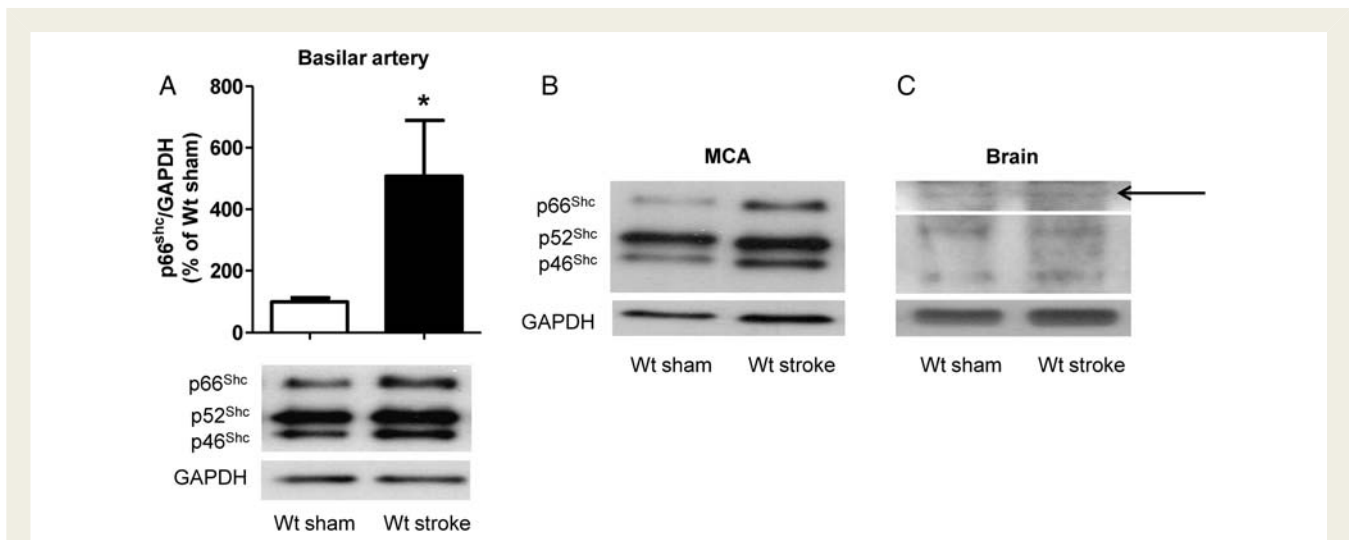
## Discussion

The present study demonstrates for the first time that genetic deletion of the adaptor protein p66<sup>Shc-/-</sup> protects mice from ischaemia/reperfusion-induced brain injury and consequent neurological deficits. This effect is paralleled by a blunted activation of the pro-oxidant enzyme NADPH oxidase, a downstream target of p66<sup>Shc</sup>, and a reduced production of free radicals.

Transient occlusion of the middle cerebral artery is a well-established model of stroke.<sup>36</sup> Indeed, in our study, this approach led to sizeable strokes and a reproducible neurological deficit. Interestingly, genetic deletion of p66<sup>Shc</sup> protected mice from ischaemia/reperfusion-induced brain injury. Following 1 h occlusion

and 24 h of reperfusion, p66<sup>Shc-/-</sup> displayed an over 50% reduction in cortical and subcortical brain lesions compared with Wt mice. Our findings expand a previous report showing that p66<sup>Shc</sup> deletion is protective against ischaemia/reperfusion injury in *ex vivo*-perfused hearts<sup>32</sup> to an *in vivo* setting on a different organ. Assessment of neurological deficit 1 h after MCAO denoted a similar degree of impairment in both Wt and p66<sup>Shc-/-</sup> mice. However, following 24 h of reperfusion, p66<sup>Shc-/-</sup> mice displayed a marked improvement in neuromotor function compared with Wt mice. This indicates that (i) the protective effects of p66<sup>Shc-/-</sup> deletion interfere primarily with reperfusion injury and not with the effects of ischaemia and (ii) that these effects lead to a better neurological recovery after brain reperfusion. In order to exclude a possible interference by different blood pressure values and/or different degree of occlusion–reperfusion, we measured blood pressure, heart rate as well as rCBF in all experimental groups. Indeed, all haemodynamic values were comparable in Wt and p66<sup>Shc-/-</sup> mice, indicating that the observed effect is not the result of gross physiological or procedural differences. These findings are of potential clinical importance, as reperfusion injury with subsequent brain oedema is an important complication after successful thrombolysis in patients presenting with ischaemic stroke.<sup>37,38</sup> Nevertheless, stroke size and neurological impairment were only examined at 24 h; although early time points are crucial, showing a preserved protective effect at later time points would be important for future developments based on the herein reported data.

Increased production of ROS is widely recognized as a key mediator of reperfusion-induced brain injury,<sup>2,9,39,40</sup> and protein carbonylation is an established marker of oxidative stress as well as the most common post-translational protein modification induced by oxidative stress.<sup>41,42</sup> Here, we report that Wt stroke mice show an increased carbonylation of brain proteins compared with Wt sham-operated mice, demonstrating that ischaemia/



**Figure 5** p66<sup>Shc</sup> protein levels in the basilar artery, middle cerebral artery, and brain. (A and B) p66<sup>Shc</sup> protein expression is increased in basilar artery and middle cerebral artery of wild-type (Wt) stroke mice compared with wild-type sham mice. (C) Protein level of p66<sup>Shc</sup> in whole-brain homogenates is hardly detectable and remains comparable between wild-type stroke and wild-type sham mice. Values are given as percentage of wild-type sham mice. \* $P = 0.0245$  for wild-type stroke vs. wild-type sham.

reperfusion injury indeed induces oxidative stress.<sup>5–8</sup> In contrast, brain tissue of p66<sup>Shc-/-</sup> stroke mice did not display increased levels of protein carbonylation compared with p66<sup>Shc-/-</sup> shams or Wt shams, suggesting that p66<sup>Shc</sup> is crucially involved in this process. In the light of the important role played by ROS in ischaemia/reperfusion-induced brain injury,<sup>39,43</sup> strategies aimed at preventing their increase following reperfusion are currently being sought for.<sup>2,39</sup> Following ischaemia, increased levels of ROS promote endothelial activation and increase the permeability of brain arteries, leading to the expression of adhesion molecules, pro-inflammatory cytokines, and increased leucocyte adhesion, all of which are recognized mechanisms influencing stroke size.<sup>39,40,43</sup> Indeed, p66<sup>Shc-/-</sup> stroke mice displayed a reduced generation of ROS and a reduced stroke size. In patients with ischaemic stroke, increased ROS levels have been measured also in circulating blood.<sup>9</sup> In line with this, we found increased levels of O<sub>2</sub><sup>-</sup> in peripheral blood of Wt stroke mice, but not in p66<sup>Shc-/-</sup> stroke mice, suggesting a systemic activation of ROS-producing pathways under these conditions. Although oxidative stress is crucially implicated in the pathogenesis of cerebrovascular disease, previous large clinical trials aimed at reducing ROS failed to improve the outcome.<sup>44,45</sup> Several factors could be taken into account to explain this failure: (i) high concentration of antioxidant supplements has been introduced in daily human diet due to significant improvement of healthcare; (ii) endogenous antioxidant defence systems may be depressed by additional antioxidant treatment; (iii) exogenous antioxidant agent may function differently from endogenous ones; and (iv) intracellular ROS production sites may be sequestered in organelles (for instance, mitochondria), thus preventing dietary antioxidants to reach their putative site of action.<sup>46</sup> Hence, the development of treatment strategies such as prevention of p66<sup>Shc</sup> activation to prevent increased

ROS production, rather than lowering it, may represent a more effective alternative.

Nicotinamide adenine dinucleotide phosphate oxidase is a membrane-bound enzyme known to be expressed in cerebral arteries<sup>47,48</sup> and recognized as a major source of ROS production in cerebrovascular disease.<sup>3,10,11</sup> Nicotinamide adenine dinucleotide phosphate oxidase expression is known to increase in disease conditions such as ischaemia,<sup>49,50</sup> and its genetic deletion in mice was shown to reduce brain infarction<sup>12</sup>; furthermore, NADPH oxidase was recently reported to be a downstream target of p66<sup>Shc</sup>.<sup>13</sup> In line with the above, protein expression of gp91phox NADPH oxidase subunit, but not of p67phox and p47phox, was strongly increased following ischaemia/reperfusion in the brain of Wt stroke mice. However, this increase was not observed in p66<sup>Shc-/-</sup> stroke mice, supporting previous reports that p66<sup>Shc</sup> is an upstream regulator of NADPH oxidase and that the p66<sup>Shc</sup>/NADPH oxidase axis is crucial for ischaemia/reperfusion-induced ROS.<sup>13</sup> To further support this associative finding, we also looked at protein expression of other anti- or pro-oxidant enzymes such as COX-2, SOD1, SOD2, and Gpx and found no significant differences in the levels of these in Wt stroke and p66<sup>Shc-/-</sup> stroke groups (data not shown). Yet, the exact mechanisms linking p66<sup>Shc</sup> and NADPH oxidase deserve further investigation.

The mammalian ShcA adaptor protein is ubiquitously expressed<sup>51–54</sup>; however, p66<sup>Shc</sup> is hardly detectable in the central nervous system,<sup>55–57</sup> with exception of the developing brain<sup>57</sup> and cultured rat neurons.<sup>58</sup> Indeed, in the present study, p66<sup>Shc</sup> protein expression was hardly detectable in whole-brain homogenates. Moreover, whole-brain p66<sup>Shc</sup> expression remained unchanged following ischaemia/reperfusion, suggesting a minor involvement of cerebral p66<sup>Shc</sup>. In sharp contrast, we found that the

basilar artery and the middle cerebral artery display higher levels of basal p66<sup>Shc</sup> expression compared with the whole brain. Furthermore, p66<sup>Shc</sup> expression in the basilar and middle cerebral arteries was dramatically increased following ischaemia/reperfusion, suggesting that cerebrovascular rather than neuronal p66<sup>Shc</sup> may be an important mediator of ischaemia/reperfusion brain injury. Our findings could be partially explained by previous reports indicating a pivotal role of the cerebral vasculature in determining stroke size. Indeed, following reperfusion, the endothelium of cerebral arteries is focally activated, thereby promoting leucocyte leakage into the extracellular matrix and inflammation both of which are important mechanisms determining neuronal damage.<sup>36,40</sup> The crucial role of cerebral arteries in determining stroke size was also recently underscored by Yin *et al.*,<sup>36</sup> who showed that vascular-specific deletion of PPAR $\delta$  increases stroke size in the mouse via an increased post-ischaemic inflammation. In further support of our interpretation is the fact that preserved endothelial function has been described as the most common mechanism protecting p66<sup>Shc-/-</sup> mice in several disease models, including diabetes,<sup>19</sup> atherosclerosis,<sup>18</sup> and ageing,<sup>20,26,59,60</sup> where ROS are known to play an important role.

In summary, this study shows for the first time that genetic deletion of p66<sup>Shc</sup> strongly reduces stroke size following ischaemia/reperfusion brain injury. In line with this, p66<sup>Shc-/-</sup> mice displayed a far milder neurological deficit following ischaemia/reperfusion compared with Wt. The observed protective effects are likely mediated by a reduced activation of the p66<sup>Shc</sup> target NADPH oxidase which leads to a decreased production of free radicals. Inhibition of this novel pathway may be a novel and effective therapeutic target in preventing reperfusion injury in patients presenting with ischaemic stroke undergoing thrombolysis of interventional reperfusion therapy.

## Study limitations

There are some limitations that need to be acknowledged and addressed regarding the present study. First of all, the use of knock out animals does not completely exclude the possibility of some adaptive mechanisms of compensatory nature taking place over the course of their life. Second, the observed blunted activation of NADPH subunits observed in p66<sup>Shc-/-</sup> stroke mice needs to be investigated further to elucidate the pathways involved. Lastly, in order to fully support our conclusions with respect to possible clinical applications, future studies including later time points as well as larger animal models and human proof-of-principle experiments should be conducted.

## Perspectives

Over the last century, an impressive increase in human life expectancy occurred; hence, also due to constant birth rates, the population is ageing. With ascending age, the incidence of cerebrovascular diseases, such as stroke, sharply increases. Although stroke is a leading cause of morbidity and mortality to date, no effective therapeutical strategy exists. In the present study, we have shown for the first time that genetic deletion of p66<sup>Shc</sup> protects mice from ischaemia/reperfusion brain injury through a blunted activation of NADPH oxidase and a reduced production of free radicals. Hence, p66<sup>Shc</sup> represents an interesting novel target to be

investigated in the context of ischaemic stroke and reperfusion injury.

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