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Neuronal HIF-1α and HIF-2α deficiency improves neuronal survival and sensorimotor function in the early acute phase after ischemic stroke

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Abstract

Hypoxia-inducible factors mediate adaptive responses to ischemia, among others, by induction of anti- and pro-survival genes. Thus, the impact of HIF on neuronal survival upon stroke is controversial. Therefore, neuron-specific knockout mice deficient for *Hif1a* and *Hif2a* were exposed to inspiratory hypoxia or ischemia–reperfusion injury. Both *Hif1a*- and *Hif2a*-deficient mice showed no altered infarct and edema size, suggesting that both HIF-α subunits might compensate for each other. Accordingly, hypoxic HIF-target gene regulation was marginally affected with exception of anti-survival *Bnip3* and pro-survival erythropoietin. In the early acute stage upon stroke, *Hif1a*/*Hif2a* double knockout mice exhibited significantly reduced expression of the anti-survival *Bnip3*, *Bnip3L*, and *Pmaip1*. Accordingly, global cell death and edema were significantly reduced upon 24 h but not 72 h reperfusion. Behavioral assessment indicated that *Hif1a*/*Hif2a*-deficient mice initially performed better, but became significantly more impaired after 72 h accompanied by increased apoptosis and reduced angiogenesis. Our findings suggest that in neurons HIF-1 and HIF-2 have redundant functions for cellular survival under ischemic conditions. By contrast, lack of anti-survival factors in *Hif1a*/*Hif2a*-deficient mice might protect from early acute neuronal cell death and neurological impairment, indicating a benefit of HIF-pathway inhibition in neurons in the very acute phase after ischemic stroke.

Keywords

Erythropoietin, hypoxia-inducible factor, ischemia, stroke, vascular endothelial growth factor

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Introduction

In response to low tissue oxygenation during ischemic stroke, cells activate hypoxia-inducible factors (HIFs) to enhance the transcription of genes involved in glycolysis, angiogenesis, and cell survival, promoting adaptation to hypoxic/ischemic stress.[1] However, also anti-survival factors have been identified as HIF-target genes.[2–4] Accordingly, results concerning the role of HIF-1 during cerebral ischemia are controversial. While Baranova et al.[5] found that neuron-specific loss of HIF-1 increases ischemic brain injury, Helton et al.[6] reported decreased tissue damage upon cerebral ischemia. Furthermore, nothing is known about the function of neuronal HIF-2.

HIFs are heterodimers consisting of an α and a β subunit. HIF-1α/HIF-1β and HIF-2α/HIF-1β dimers are the primary factors regulating hypoxic transcriptional responses in most mammalian cells. HIF-1α and HIF-2α exhibit similar functional domain structures, containing DNA binding and dimerization domains at their N termini and transactivation domains at their C termini.[7] HIFs are primarily regulated by changes in protein stability and transcriptional activity in an oxygen-dependent manner. Under normoxic conditions, both α subunits are hydroxylated

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on conserved proline and asparagine residues by the family of prolyl-4-hydroxylase domain (PHD) proteins and factor inhibiting HIF (FIH), respectively, whose activity is dependent on molecular oxygen, ferrous iron, 2-oxoglutarate, and reducing compounds like ascorbate. While prolyl-4-hydroxylation results in recruitment of the von Hippel-Lindau protein E3 ubiquitin ligase and immediate proteasomal degradation of HIF-1α and -2α, asparaginyl hydroxylation blocks interaction with the transcriptional co-activators p300 and CBP, reducing its transcriptional activity.[1,8–12] By contrast, when oxygenation declines during hypoxia or ischemia, PHDs and FIH are less active. As a result, hypohydroxylated HIF-α accumulates and translocates into the nucleus to form the HIF complex by dimerization with HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT). Subsequently, HIF specifically binds to hypoxia response elements (HREs) in the promoters and enhancers of numerous genes followed by recruitment of p300/CBP resulting in enhanced transcription of HIF-responsive genes.[1,8–12]

A growing number of pre-clinical studies in rodents strongly suggests that activation of the HIF signaling pathway prior or shortly after ischemic stroke by application of low-molecular weight hydroxylase inhibitors[5,13–19] or brief exposure to mild systemic hypoxia,[20–23] reduces tissue damage and increases functional recovery from ischemic stroke. However, as these therapeutic strategies unavoidably lead to pleiotropic activation of HIF signaling throughout all cell types of the central nervous system (CNS), it is difficult to draw conclusions about the definite significance of the HIF signaling pathway in neurons and their viability upon ischemia–reperfusion injury. Furthermore, although HIF-1α and HIF-2α are closely related and share numerous target genes, they also regulate unique target genes, suggesting that HIF-1α and HIF-2α may exhibit distinct roles in the neuronal response to ischemic conditions. Thus, in the present study we generated transgenic mice bearing neuron-specific deficiencies of *Hif1a* and/or *Hif2a*, and studied the histological and functional outcome upon mild and severe focal cerebral ischemia.

Materials and methods

Animal experiments

All animal experiments were approved by the local animal welfare committee (Regierungspräsidium Karlsruhe, Germany, approval number: 35-9185.81/G-103/12, 35-9185.81/G-210/14), conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were performed in accordance with the recently published Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (http://www.nc3rs.org/ARRIVE). All mice used in the animal experiments were randomized. The operator or investigator was blinded for the respective mouse genotypes throughout the study. Evaluation of all read-out parameters was done independently and in a blinded fashion.

Generation of neuron-specific Hif1a, Hif2a, and Hif1a/Hif2a knockout mice

All mice were maintained at the animal facility of the University of Heidelberg under specific pathogen-free conditions, a controlled 12:12 h light-dark cycle, constant room temperature (22 ± 2°C) with food and water *ad libitum*. $Hif1a^{flox/flox}$ [24] and $Hif2a^{flox/flox}$ mice[25] were obtained from Dr. Ben Wielockx (Department of Clinical Pathobiochemistry, Technical University of Dresden). $Hif1a^{f/f}$ and $Hif2a^{f/f}$ mice were intercrossed to generate $Hif1a/Hif2a^{ff/ff}$ mice. Subsequently, $Hif1a^{f/f}$, $Hif2a^{f/f}$, and $Hif1a/Hif2a^{ff/ff}$ mice were bred with a transgenic mouse line expressing Cre recombinase under control of the Ca^{2+}/calmodulin-dependent protein kinase IIα promotor (*CaMKIIα:cre*)[26,27] to obtain homozygous forebrain neuron-restricted knockout mice ($nHif1a^{\Delta/\Delta}$, $nHif2a^{\Delta/\Delta}$, $nHif1a/Hif2a^{\Delta\Delta/\Delta\Delta}$). All mouse strains were at least nine generations backcrossed to C57BL/6. Mice were genotyped using the primers (Eurofins Genomics, Ebersberg, Germany) described in Table S1.

Middle cerebral artery occlusion

Male mice (8–10 weeks old) were anesthetized by a mixture of 2% isoflurane, 70% N_2O and remainder O_2, and were maintained by reducing the isoflurane concentration to 1.0–1.5%. Core body temperature was maintained at 37°C throughout surgery by using a feedback-controlled heating device. To induce focal cerebral ischemia, a 7-0 silicon rubber-coated nylon monofilament (Doccol Corporation, Redlands, CA, USA) was introduced into the left internal carotid artery (ICA) and pushed toward the left MCA as described.[27] The intraluminal suture was left for 30–60 min. Then, animals were re-anesthetized and the occluding monofilament was withdrawn to allow reperfusion for 24–72 h. Subsequently, animals were sacrificed by decapitation, and brains were removed and embedded into Tissue-Tek (Sakura Finetek, Staufen, Germany) for histological analyses. From each brain, 24 coronal cryosections (10 µm thickness; 0.4 mm distance) were prepared and submitted to Nissl staining for quantification of infarct and edema size as described previously.[17,27] Briefly, brain slices were digitized, and infarct and edema volume was

measured using the image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA). Animals that met the following criteria were excluded from end-point analyses: (i) death after induction of middle cerebral artery occlusion (MCAO) and (ii) intracerebral hemorrhage (macroscopically assessed during brain sampling; Table S2).

Systemic hypoxia

Mice (10–14 weeks old) were exposed to normobaric hypoxia at 6% oxygen (corresponding to 9500 m altitude) for 6 h or were kept at room air pressure (~21% oxygen). During the entire procedure mice had free access to food and water. Hypoxia was achieved by substituting nitrogen for oxygen using a Digamix 5SA 18/3 a pump (H. Wösthoff, Bochum, Germany) as described.[28] Then, mice were sacrificed by decapitation, brains were isolated, shock frozen in liquid nitrogen and stored at −80°C until use.

Assessment of functional outcome

Sensorimotor function was assessed by using the corner test and the latency to move test as described previously.[29,30] Details are specified in the Supplementary Information. In addition, neurological function before and after MCAO was studied using a modified Bederson neurological deficit score,[31] according to the following scoring system: 0, no deficit; 1, whole body bent upon hanging from tail; 2, forelimb flexion; 3, unidirectional turning; 4, unidirectional circling; 5, no movement.

Analysis of cerebrovascular anatomy

Mice were sacrificed by CO_2 asphyxiation, the skin and the ribs were cut at the midline of the chest, and the thorax cavity was opened. The right atrium was incised to allow venous outflow followed by insertion of the perfusion cannula into the left ventricle. Animals were perfused at a rate of ~10–15 ml/min with pre-warmed Ringer's solution containing 0.1 mM sodium nitroprusside (Sigma-Aldrich, Steinheim, Germany) and 0.1 mM adenosine (Sigma) until blood was completely replaced by Ringer's solution. Then, perfusion was continued with 10 ml of a pre-warmed gouache pigment solution that was prepared as described previously.[32] Subsequently, brains were carefully harvested and incubated overnight in 4% paraformaldehyde at 4°C, and digital images were taken under a dissecting microscope.

TdT-mediated dUTP nick-end labeling assay

Apoptotic cells were detected in brain sections using the TdT-mediated dUTP nick-end labeling (TUNEL) assay. Details are specified in the Supplementary Information.

Immunofluorescence staining, image acquisition, and analysis

Immunofluorescent staining was used to detect endothelial cells and microglia/macrophages in brain sections. For a detailed description see the Supplementary Information.

RNA isolation and real-time polymerase chain reaction

A detailed description can be found in the Supplementary Information. Oligonucleotide primers were purchased from Eurofins Genomics (Ebersberg, Germany; for primer sequences, see Table S3).

Protein extraction and immunoblotting

A detailed description is provided in the Supplementary Information.

Statistical analysis

If not stated otherwise, the results are presented as mean + standard error of the mean (SEM). Kolmogorov–Smirnov test ($n = 5$–7) and D'Agostino-Pearson omnibus test ($n \geq 8$) were applied to analyze data sets for Gaussian distribution. Grubb's test was used on normal distributed data sets ($n \geq 5$) to identify statistical outliers (http://www.graphpad.com/quickcalcs/grubbs1/). Unpaired and paired two-tailed Student's t-test or two-way analysis of variance (ANOVA) combined with Holm-Sidak's multiple comparison test were applied to determine statistical significance. Prism 6 (GraphPad Software, San Diego, CA, USA) was used for data plotting and statistical analyses.

Results

Characterization of neuron-specific Hif1a and Hif2a knockout mice

In the forebrain of $nHif1a^{\Delta/\Delta}$ mice, *Hif1a* mRNA was markedly reduced by 80% in comparison to wild-type (WT) littermates (Figure 1(a)). Accordingly, hypoxia-induced stabilization of HIF-1α protein in the forebrain was absent, as hypoxic HIF-1α levels were significantly reduced by about 90% in $nHif1a^{\Delta/\Delta}$ mice (Figure 1(a)), suggesting that neurons are the main cellular source of HIF-1α in the CNS. Our data further reveal that neuron-restricted *Hif1a* knockout did not cause a

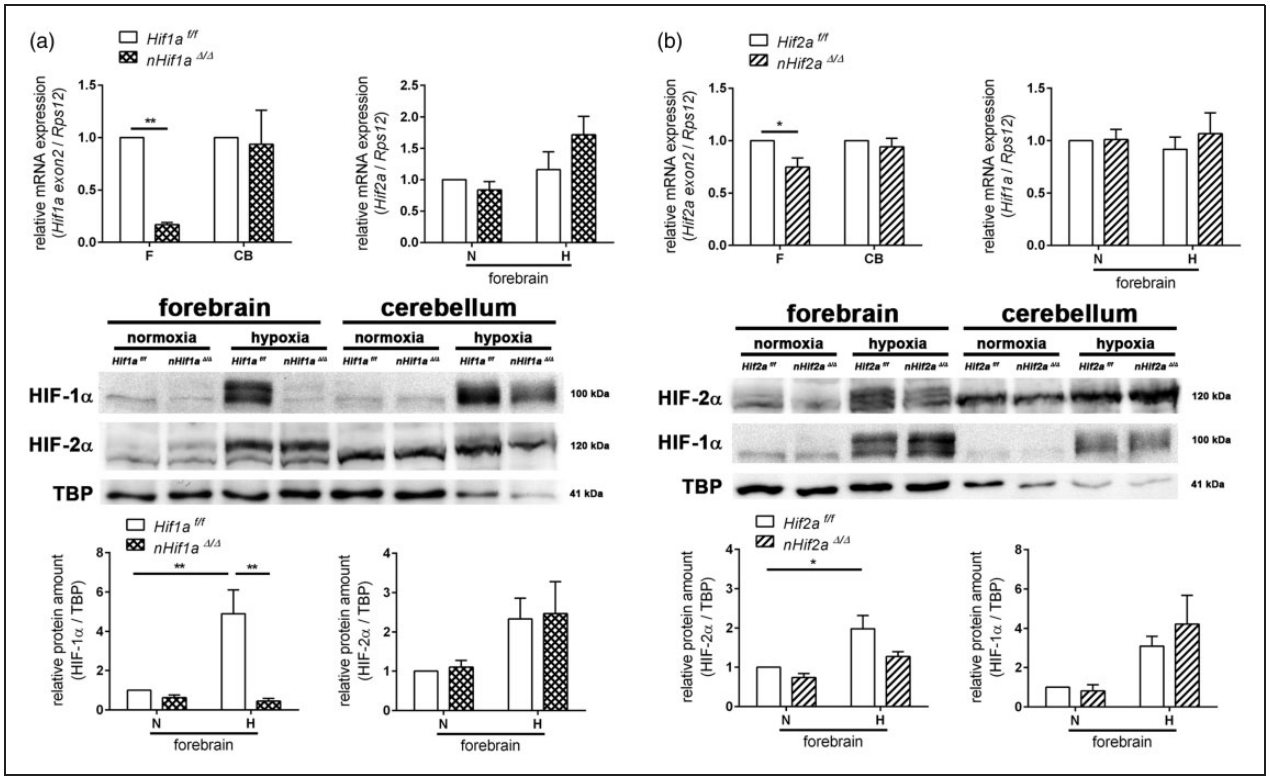

Figure 1. Characterization of neuron-specific *Hif1a* and *Hif2a* knockout. *Hif1a*$^{f/f}$ and *nHif1a*$^{\Delta/\Delta}$ (a) or *Hif2a*$^{f/f}$ and *nHif2a*$^{\Delta/\Delta}$ (b) mice were exposed to normobaric hypoxia (6% oxygen) for 6 h or were kept at normoxic conditions. Nuclear proteins and RNA were prepared from forebrain and cerebellum. Gene expression was determined by real-time polymerase chain reaction (PCR) ($n = 5$, per group) and Western blotting ($n = 3$–4, per group). Values are normalized to *Rps12* (mRNA) or TBP (protein) and expressed as fold change to normoxic *Hif1a*$^{f/f}$ and *Hif2a*$^{f/f}$, respectively. Significant differences determined by two-way ANOVA with Holm-Sidak post-hoc test are indicated with *($p < 0.05$) or **($p < 0.01$).
CB: cerebellum; F: forebrain; H: hypoxia; N: normoxia.

compensatory up-regulation of *Hif2a* mRNA and protein in the forebrain of animals kept under normoxic or hypoxic conditions (Figure 1(a)). By contrast, neuronal ablation of *Hif2a* reduced total *Hif2a* mRNA in the murine forebrain by only 25% (Figure 1(b)). At protein level, HIF-2α in the forebrain of *nHif2a*$^{\Delta/\Delta}$ mice exposed to systemic hypoxia was decreased by roughly 35% as compared to WT animals under the same conditions and was not significantly different from normoxic levels (Figure 1(b)). These results together with a previous study indicate that HIF-2α in brain tissue predominantly derives from non-neuronal cells such as astrocytes and endothelial cells.[33] Under both normal and declined oxygen tensions, *Hif1a* gene expression was not significantly different between WT and *nHif2a*$^{\Delta/\Delta}$ mice (Figure 1(b)).

Reduced neuronal HIF-1α or HIF-2α levels do not affect infarct or edema size during ischemic stroke

Next, we subjected mice to either 60 min or 30 min of MCAO provoking severe or mild brain tissue damage, respectively. Following one or three days of reperfusion, global infarct and edema size were histologically determined. Irrespective of mild or severe ischemic stroke, infarct and edema volume of *nHif1a*$^{\Delta/\Delta}$ mice was comparable to that obtained for WT animals (Figure 2(a) and (b), Table S4). Similarly, neuronal *Hif2a* deficiency did not affect the lesion or edema size in mice subjected to either mild or severe cerebral ischemia (Figure 2(a) and (b), Table S4). Despite similar lesion size, WT animals undergoing 30 min of MCAO showed an increased, but non-significant, mortality rate during prolonged reperfusion phase as compared to *nHif1a*$^{\Delta/\Delta}$ and *nHif2a*$^{\Delta/\Delta}$ mice, respectively (Table S2, Figure S1).

Neuronal HIF-α deletion maintains hypoxic gene induction but shows subunit dependency

HIF-independent infarct and edema size might be due to a mutual compensation in *Hifa* single knockout mice. Alternatively, it may also result from a compensatory co-expression of both pro- and anti-survival

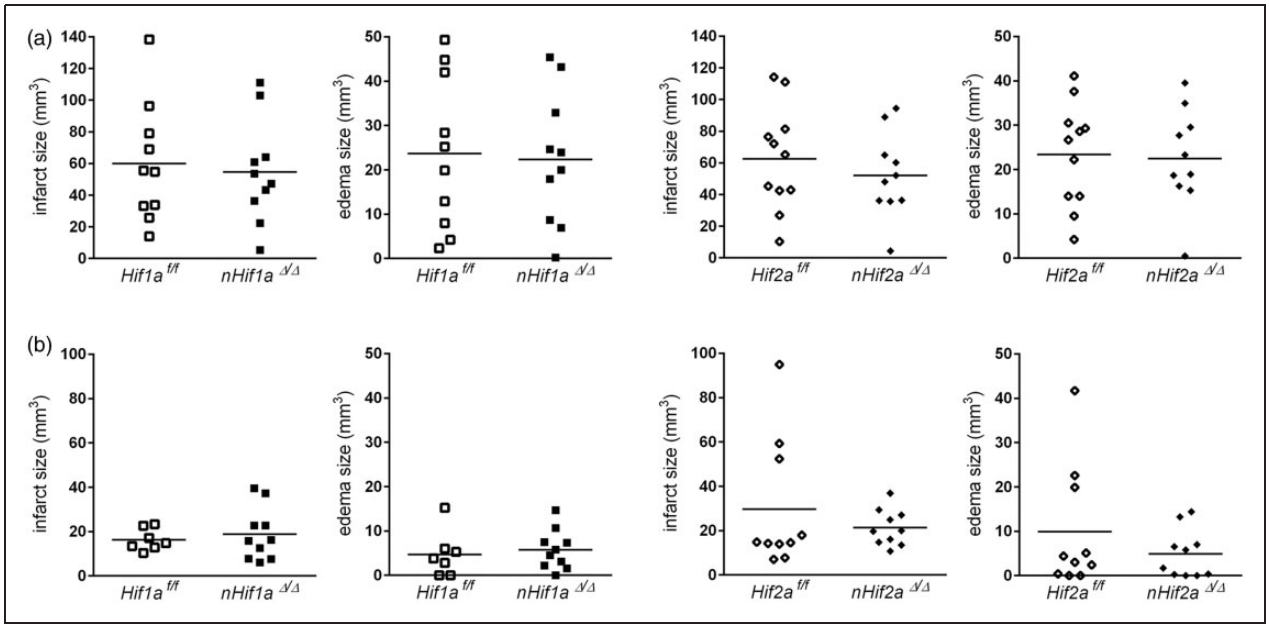

Figure 2. Neuronal *Hif1a* or *Hif2a* deficiency does not affect global brain tissue injury following mild or severe ischemic stroke. Mice were subjected to transient focal ischemic stroke: (a) 60 min of MCAO followed by 24 h reperfusion ($n = 10$–11, per group) or (b) 30 min of MCAO followed by 72 h reperfusion ($n = 7$–10, per group). Brains were removed and from each brain, 24 coronal cryosections (10 μm thickness; 0.4 mm distance) were prepared and submitted to cresyl violet staining for quantification of infarct and edema size. Unpaired two-tailed t-test was applied to determine statistical significance.

HIF-target genes after a hypoxic/ischemic stimulus. To address the latter, we analyzed the expression of HIF-regulated anti-survival genes such as *Bnip3* and *Pmaip1* (also known as *Noxa*),[2–4] and compared them to prominent protective factors such as vascular endothelial growth factor (*Vegf*) and erythropoietin (*Epo*), which not only directly protect neurons upon cerebral ischemia through antiapoptotic pathways, but also contribute to long-term regeneration via induction of angiogenesis and neurogenesis,[34,35] and to *Glut-1 and Glut-3*, which increase glucose transport.[36,37] Overall, hypoxic induction of anti- and pro-survival factors was comparable. Irrespective of genotype, exposure to a short episode of cerebral hypoxia resulted in significant up-regulation of *Vegf* and *Epo* within the forebrain, and also *Glut-1* and *Glut-3* expression was moderately enhanced after cerebral hypoxia (Figure 3(a)). On the other hand, also anti-survival genes were induced to a similar degree. Cerebral hypoxia enhanced *Pmaip1* mRNA expression irrespective of genotype and *Bnip3* transcription was increased by 50% in hypoxic WT mice (Figure 3(b)), suggesting that pro- and anti-survival factors could indeed lead to a mutual counterbalance.

With respect to redundant HIF-α function, some differences were seen. Although normoxic and hypoxic *Vegf* as well as *Glut-1* and *Glut-3* expression was not significantly altered in both $nHif1a^{\Delta/\Delta}$ or $nHif2a^{\Delta/\Delta}$ mice in comparison to WT animals (Figure 3(a)), *Epo* and *Bnip3* showed a clear HIF-α subunit dependency. Upon hypoxia, neuronal loss of *Hif1a* caused a significant increase in total *Epo* mRNA expression, while loss of *Hif2a* resulted in reduced expression as compared to WT controls (Figure 3(a)). It suggests that similar to other cell types,[38] neuronal *Epo* transcription is preferentially controlled by HIF-2 and not HIF-1. Basal expression of *Bnip3* and *Pmaip1* was not significantly different in both $nHif1a^{\Delta/\Delta}$ and $nHif2a^{\Delta/\Delta}$ mice as compared to WT animals (Figure 3(b)). Although hypoxic up-regulation of *Bnip3* was comparable in $nHif2a^{\Delta/\Delta}$, it was completely absent in $nHif1a^{\Delta/\Delta}$ mice, suggesting that neuronal *Bnip3* expression is regulated by HIF-1 rather than HIF-2 (Figure 3(b)).

In the cerebellum of both *Hifa* knockout mouse lines, even under hypoxic conditions, mRNA expression of all studied genes was comparable to that obtained in WT littermates (Figure S2), reflecting low recombination efficiency due to low *CaMKIIα* promoter activity in this tissue. Overall, these data support our hypothesis that HIF-1α and HIF-2α functions in neurons are to a certain degree redundant.

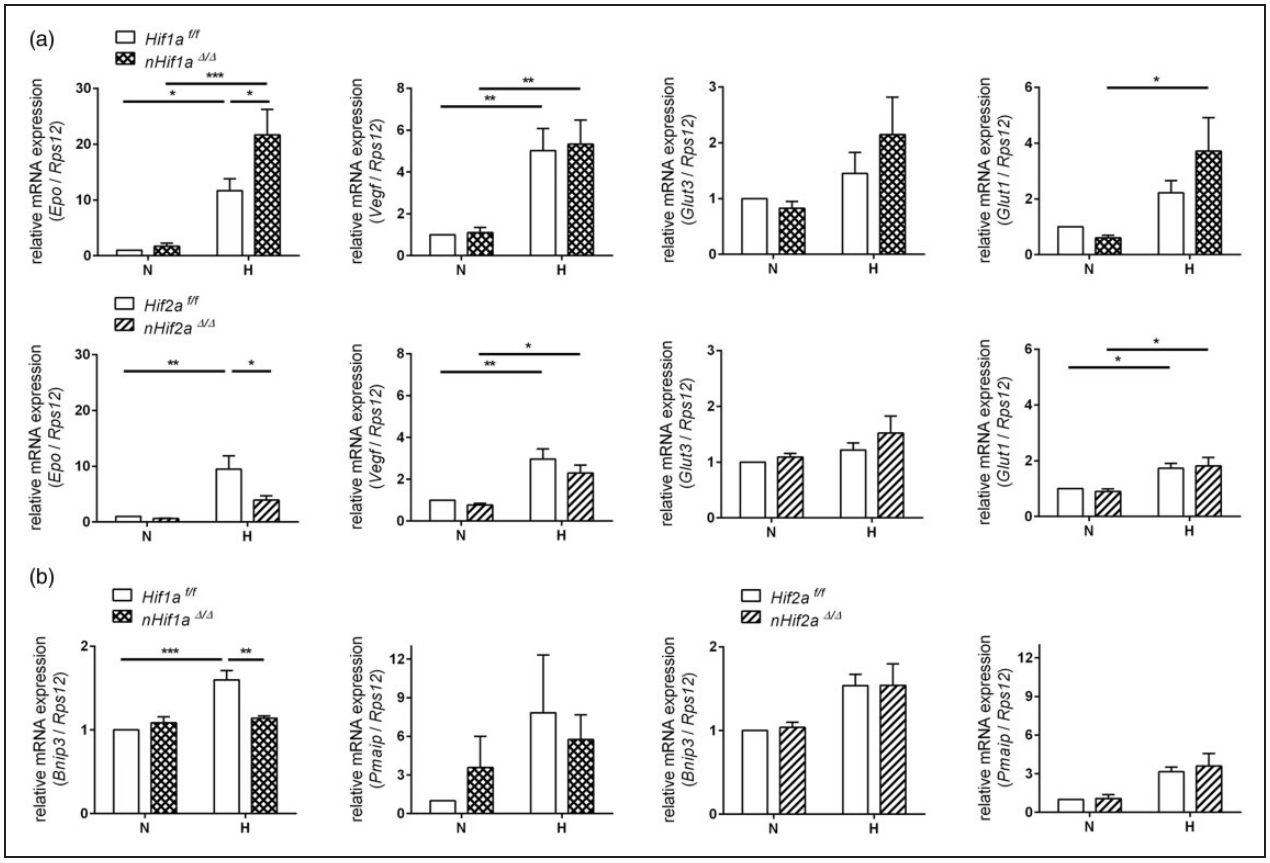

Figure 3. Expression of HIF-targeted genes in the forebrain of neuron-specific *Hif1a* and *Hif2a* knockout mice. *Hif1a*$^{f/f}$, *nHif1a*$^{\Delta/\Delta}$, *Hif2a*$^{f/f}$, and *nHif2a*$^{\Delta/\Delta}$ mice ($n = 5$, per group) were exposed to normobaric hypoxia (6% oxygen) for 6 h or were kept at normoxic conditions. Expression of (a) pro-survival and (b) anti-survival HIF-target genes within forebrain was analyzed by real-time PCR. Values are normalized to *Rps12* mRNA and expressed as fold change to normoxic *Hif1a*$^{f/f}$ and *Hif2a*$^{f/f}$, respectively. Significant differences determined by two-way ANOVA with Holm-Sidak post-hoc test are indicated with *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$). H: hypoxia; N: normoxia.

Inactivation of HIF-1 and HIF-2 in neurons partially impairs expression of oxygen sensitive genes in hypoxic brain tissue

In order to circumvent potential HIF-α redundancy, we generated neuron-specific *Hif1a*/*Hif2a* double knockout mice, and studied HIF-target gene expression within the forebrain. Similar to *Hifa* single knockout, basal expression of all studied genes was not significantly different between *nHif1a*/*Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice and WT animals (Figure 4). However, *Vegf* and *Bnip3* upregulation found in WT upon cerebral hypoxia was completely abrogated in *nHif1a*/*Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice (Figure 4), indicating neurons as major CNS source for both factors upon global hypoxia. By contrast, hypoxic mRNA levels of *Epo* (7.9 vs. 5.7, $p = 0.446$), *Glut-1* (2.8 vs. 1.9, $p = 0.142$), *Glut-3* (1.4 vs. 1.1, $p = 0.608$), and *Pmaip1* (4.4 vs. 4.5, $p = 0.954$) were not or only slightly attenuated in forebrains of hypoxic *nHif1a*/*Hif2a*$^{\Delta\Delta/\Delta\Delta}$ animals when compared to WT littermates (Figure 4). This might reflect the contribution of other resident brain cells like astrocytes, endothelial cells, oligodendrocytes, or microglia to total mRNA levels of these genes during the hypoxic adaptive response.

Neuronal loss of HIF-1 and HIF-2 decreases infarct and edema size in the early acute stage of mild ischemic stroke

We next investigated whether attenuation of the HIF pathway in neurons by cell type-specific knockout of both *Hif1a* and *Hif2a* influences global brain damage upon mild or severe ischemic stroke. Upon 60 min of MCAO and 24 h reperfusion, massive cell death occurred within cortical and striatal brain regions, and a pronounced hemispheric swelling was determined. Under these conditions, quantification of global lesion and edema volume did not reveal significant differences between *Hif1a*/*Hif2a*$^{ff/ff}$ and *nHif1a*/

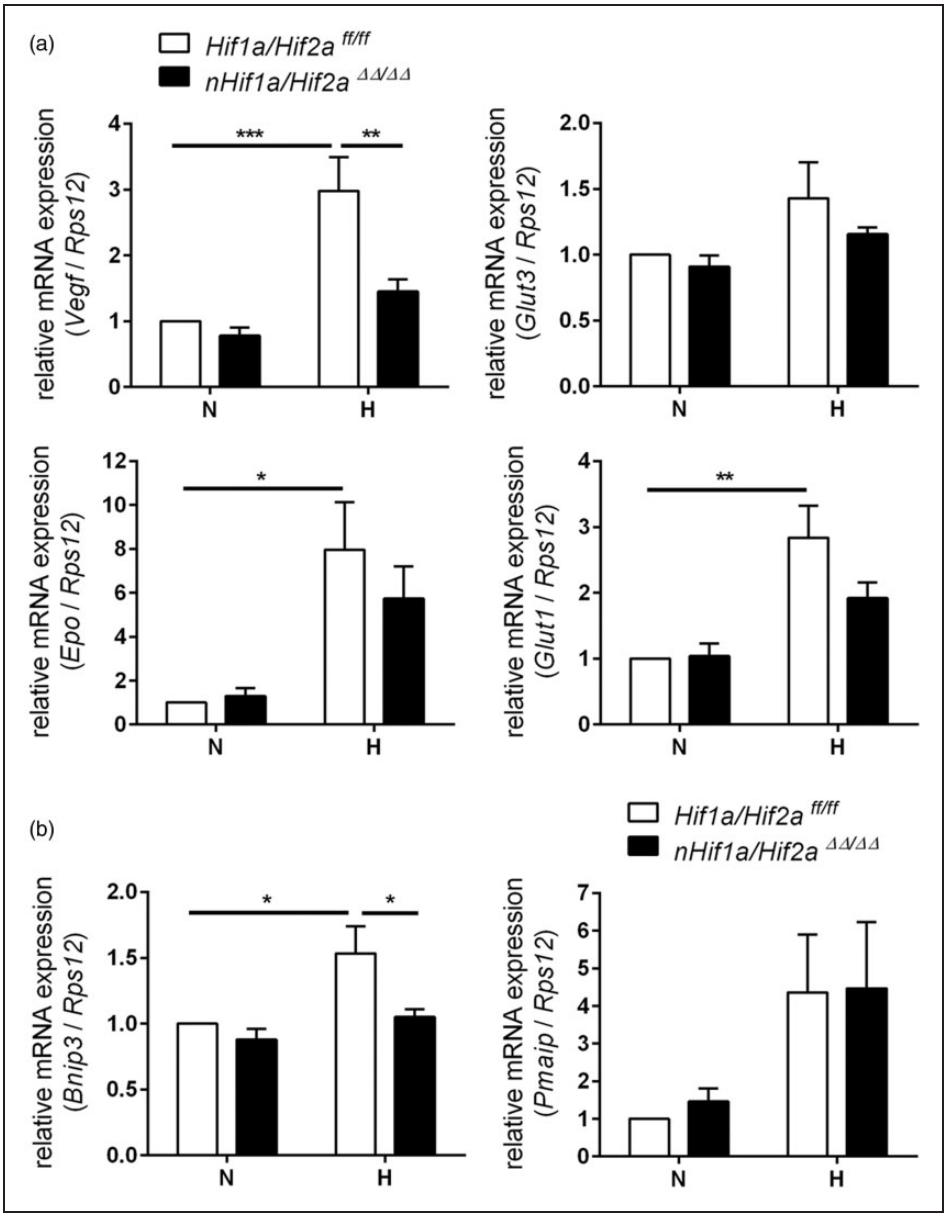

Figure 4. Expression of HIF-regulated genes in the forebrain of neuron-specific *Hif1a/Hif2a* knockout mice. *Hif1a/Hif2a*$^{ff/ff}$ and *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice ($n = 5$, per group) were exposed to normobaric hypoxia (6% oxygen) for 6 h or were kept at normoxic conditions. Expression of (a) pro-survival and (b) anti-survival HIF-target genes within forebrain was analyzed by real-time PCR. Values are normalized to *Rps12* mRNA levels and expressed as fold change to normoxic *Hif1a/Hif2a*$^{ff/ff}$. Significant differences determined by two-way ANOVA with Holm-Sidak post-hoc test are indicated with *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$).
H: hypoxia; N: normoxia.

Hif2a$^{\Delta\Delta/\Delta\Delta}$ mice (Figure 5(a), Table S4). In animals subjected to 30 min of MCAO and up to 72 h reperfusion, the loss of cells was almost restricted to the striatum, and edema formation was moderate. Upon 24h of reperfusion, both infarct and edema size were significantly reduced in *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice as compared to WT (Figure 5(b), Table S4). By contrast, in the acute (72 h after onset of reperfusion) stage, global lesion and edema volume were similar in *Hifa* deficient and control mice (Figure 5(b), Table S4). Furthermore, a non-significant tendency toward reduced post-stroke survival was determined in *Hif1a/Hif2a*$^{ff/ff}$ mice as compared to *Hifa* deficient animals (Table S2, Figure S1). In order to analyze whether the lack of neuronal HIF activity affects the cerebrovascular anatomy which might either enhance or aggravate tissue injury upon MCAO, the cerebral vasculature at the circle of Willis was visualized by pigment particle

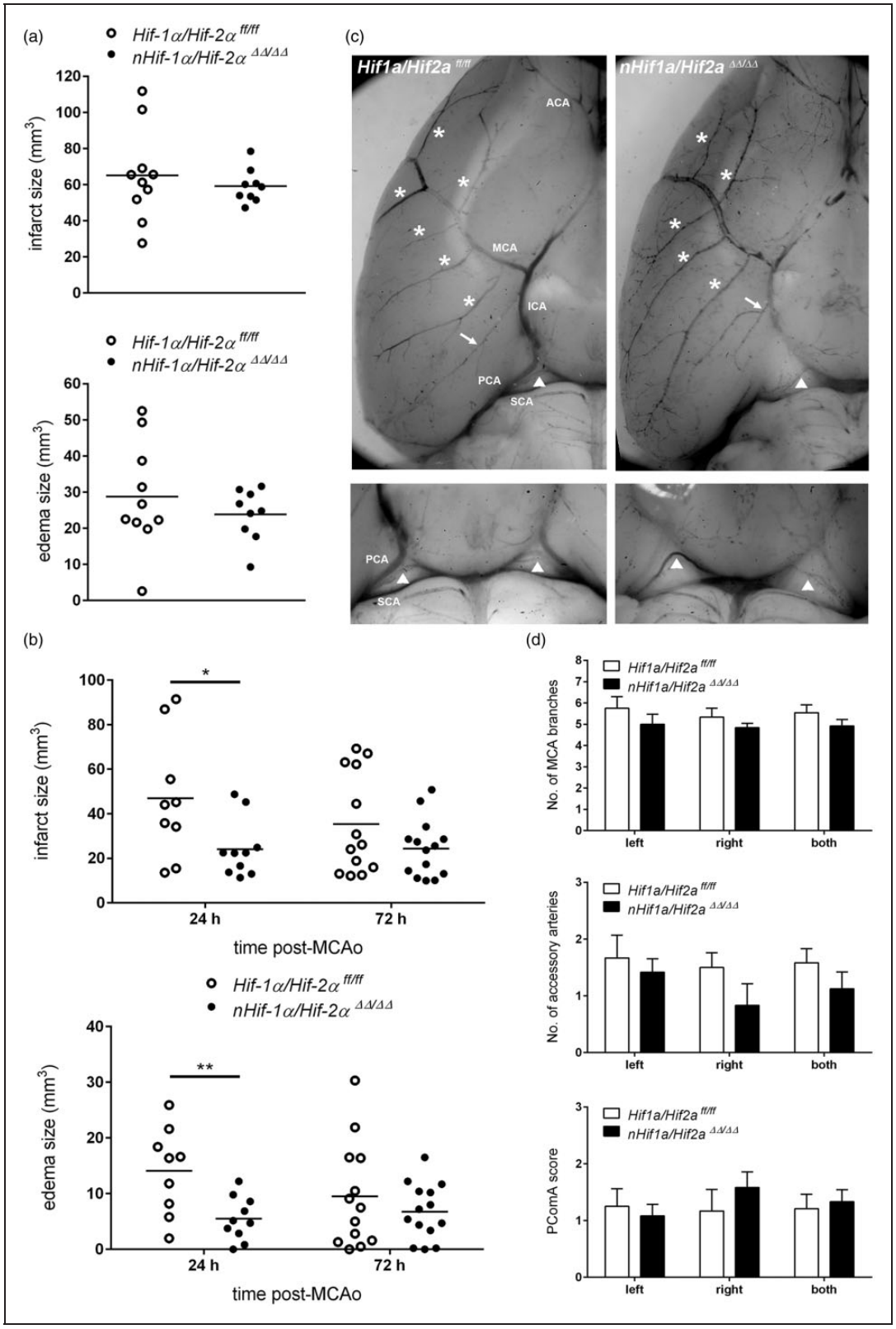

Figure 5. Neuron-restricted ablation of *Hif1a* and *Hif2a* decreases global damage of cerebral tissue in the early acute phase after mild ischemic stroke, which is not due to altered cerebrovascular architecture. Mice underwent transient focal ischemic stroke using different conditions: (a) 60 min of MCAO followed by 24 h reperfusion ($n = 9$–10, per group) or (b) 30 min of MCAO followed by 24 h ($n = 9$–10, per group) and 72 h ($n = 13$–14, per group) reperfusion, respectively. Brains were removed, coronal cryosections were

(continued)

perfusion (Figure 5(c)). The number of arterial branches that arise from the MCA main trunk was comparable between WT and *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice (Figure 5(c) and (d)). In most of the animals, we also found accessory arteries that arise near the MCA from the ICA, and potentially supply some of the MCA territory. In comparison to *Hif1a/Hif2a*$^{ff/ff}$ mice, the number of accessory arteries was not statistically different in *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice (Figure 5(c) and (d)). In our experimental model of focal cerebral ischemia, a nylon monofilament is introduced in the external carotid artery and is advanced through the ICA until blood flow to the MCA is impeded, and may also lead to hypoperfusion of regions (e.g. hippocampus and thalamus) supplied by the posterior cerebral artery (PCA). However, if the blood flow from the carotid circulation is shut down, the ipsilateral PCA could be supplied by the superior cerebellar artery (SCA) through the posterior communicating artery (PComA). Development of the PComA is highly variable in mice from different strains and within the same strain ranging from complete absence to bilateral presence, and from hypo to hyper plasticity.[39–41] Along this line, in mice lacking functional PComA, the PCA territory has an enhanced susceptibility to focal ischemia caused by the intraluminal filament technique.[40,41] Thus, we evaluated the PComA plasticity according to the following scoring system: 0, no anastomosis between PCA and SCA; 1, hypoplastic PComA; 2, well-developed PComA. Although we determined inter-individual differences in accordance with previously published studies, the mean PComA score in *Hifa* deficient and control mice was quite comparable (Figure 5(c) and (d)).

Blockage of HIF pathway in neurons improves sensorimotor function in the early acute phase, while it impairs functional outcome in the acute stage of mild ischemic stroke

We tested sensorimotor and general neurological function of *Hif1a/Hif2a*$^{ff/ff}$ and *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice suffering from mild ischemic stroke. Irrespective of genotype, mice studied with corner test before induction of stroke turned equally to the left and right side. However, in both the early acute (24 h after onset of reperfusion) and acute (72 h after onset of reperfusion) stage of stroke, WT mice turned preferentially to the ipsilateral left side, which is typically not affected by paralysis (Figure 6(a), Table S5). By contrast, *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice showed no side preference in the early acute phase similar to pre-testing results, pointing to an early protective effect of HIF deficiency. However, later on the behavior worsened, and the animals now turned mainly to the ipsilateral left side (Figure 6(a), Table S5). These results were sustained by the latency to move test, demonstrating that WT but not *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice showed a strongly reduced motor activity at the early acute stage as compared to the pre-test (Figure 6(b), Table S5). However, in the acute phase motor activity in WT animals returned to baseline, while latency to move markedly increased in *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice (Figure 6(b), Table S5). Similarly, at the acute stage *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice exhibited a significantly higher neurological deficit score in comparison to WT (Figure 6(c), Table S5). Accordingly, early body weight loss as result of stroke was significantly attenuated in *Hifa* deficient mice (Figure 6(d), Table S5). Later, body weight remained constant in WT mice, but further decreased in *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice (Figure 6(d), Table S5).

Overall, our functional evaluation suggests that blockage of the HIF pathway in neurons improves early acute outcome from ischemic stroke, while it impairs neuronal recovery in the acute phase.

Neuronal loss of HIF-1 and HIF-2 reduces expression of anti-survival genes in the early acute phase, but increases apoptotic cell death and decreases angiogenesis in the acute phase of mild ischemic stroke

While neurons predominantly undergo necrotic cell death in the early acute phase, the frequency of

Figure 5. Continued

prepared and submitted to cresyl violet staining for quantification of infarct and edema size. Significant differences determined by unpaired two-tailed Student's t-test are indicated with *($p < 0.05$) or **($p < 0.01$). (c,d) In adult *Hif1a/Hif2a*$^{ff/ff}$ and *nHif1a/Hif2a*$^{\Delta\Delta/\Delta\Delta}$ mice ($n = 6$, per group), the cerebral vasculature was stained through transcardial pigment particle perfusion. For each mouse arterial branches (labeled with asterisks) that arise from the left or right MCA trunk as well as accessory arteries (labeled with arrows) that run more or less parallel to the course of the left or right MCA were quantified. The PCA can be supplied by the SCA through the PComA (labeled with arrowheads). Accordingly, PComA plasticity was scored on scale of 0 to 2 with 0 = no anastomosis between PCA and SCA, 1 = hypoplastic PComA, and 2 = well-developed PComA. Two-way ANOVA with Holm-Sidak's multiple comparison test was applied to determine statistical significance.

ACA: anterior cerebral artery; ICA: internal carotid artery; MCA: middle cerebral artery; PCA: posterior cerebral artery; PComA: posterior communicating artery; SCA: superior cerebellar artery.

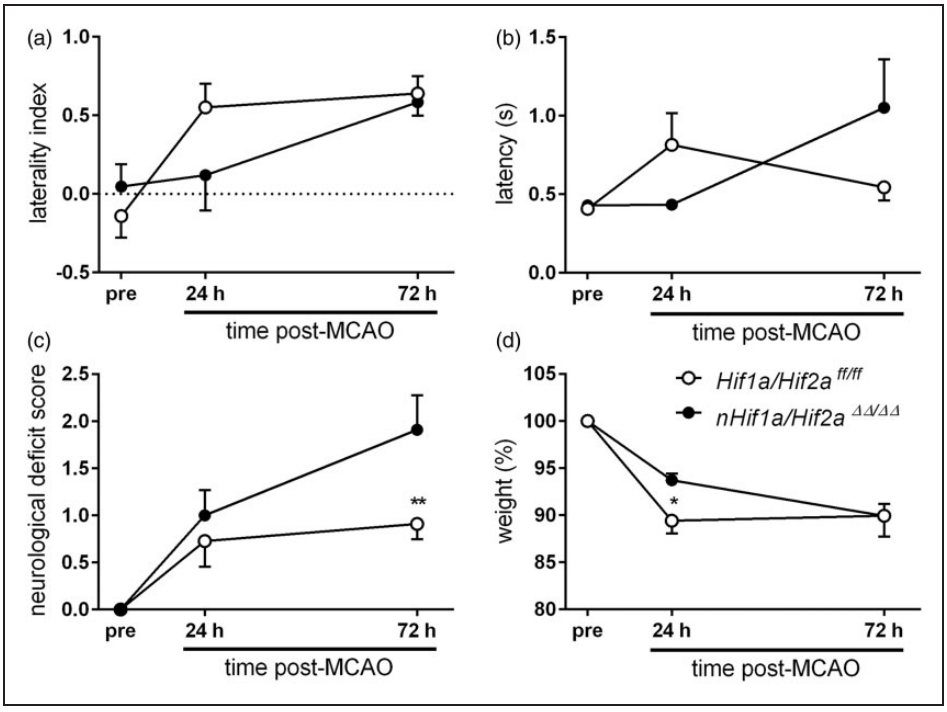

Figure 6. Neuron-specific *Hif1a/Hif2a* knockout mice show improved and impaired sensorimotor function in the early acute and acute phase after mild ischemic stroke, respectively. Mice were subjected to 30 min of MCAO followed by 72 h reperfusion ($n = 13$–14, per group). Neurological function was assessed 24 h before, and 24 and 72 h after transient MCAO by using (a) Corner test, (b) Latency to move test, and (c) a modified Bederson neurological deficit score. (a) The laterality index (LI) was calculated according to the formula: LI = (turns to the left side – turns to the right side)/total number of turnings. (d) shows the body weight before, 24 and 72 h after onset of reperfusion. Significant differences determined by two-way repeated measures ANOVA with Holm-Sidak post-hoc test are indicated with *($p < 0.05$) or **($p < 0.01$).

apoptosis strongly increases in acute stages of ischemic stroke. Thus, we analyzed whether the observed changes in behavior in *Hifa* deficient mice may correlate with the apoptosis rate after cerebral ischemia. In the early phase, in animals subjected to 30 min of MCAO and 24 h reperfusion, the number of apoptotic cells within the entire infarcted tissue was slightly reduced in $nHif1a/Hif2a^{\Delta\Delta/\Delta\Delta}$ mice (Figure 7(a)). Sub-regional analysis revealed decreased apoptosis in ipsilateral cortex (55 ± 18 vs. 170 ± 54 cells/mm^2, $p = 0.069$), but not striatum (422 ± 24 vs. 476 ± 79 cells/mm^2, $p = 0.564$) of *Hif1a/Hif2a* double knockout as compared to $Hif1a/Hif2a^{ff/ff}$ mice. However, at later stages, i.e. 72 h after mild ischemic stroke, apoptotic cell death within the entire infarct area was significantly higher in $nHif1a/Hif2a^{\Delta\Delta/\Delta\Delta}$ mice (Figure 7(a)). Sub-regional quantification in cortex (239 ± 18 vs. 165 ± 34 cells/mm^2, $p = 0.081$) and striatum (438 ± 75 vs. 310 ± 15 cells/mm^2, $p = 0.124$) revealed a likewise contribution of both areas for enhanced apoptosis in *Hifa* deficient animals. We further showed that the number of microglia/macrophages was not significantly different between WT and *Hifa* deficient mice during both early acute and acute stages, suggesting that the enhanced number of apoptotic cells in brains of $nHif1a/Hif2a^{\Delta\Delta/\Delta\Delta}$ mice is not due to attenuated phagocytosis through activated microglia/macrophages (Figure 7(b)). In order to identify potential underlying molecular mechanisms, we investigated the expression of anti- and pro-survial HIF-target genes in *Hif1a/Hif2a* deficient mice and WT littermates upon mild cerebral ischemia; 6 h after the onset of reoxygenation, the anti-survival HIF-target genes *Bnip3*, *Bnip3L*, and *Pmaip1* as well as *Vegf* were significantly up-regulated in the ipsilateral hemisphere of WT animals (Figure 7(d)). In *Hif1a/Hif2a* double knockout mice, the induction of *Bnip3*, *Bnip3L* (also known as *Nix*),[42] and *Pmaip1* in ischemic brain tissue was only marginal, and significantly lower as those in WT (Figure 7(d)). In contrast to the attenuated *Vegf* expression in *Hif1a/Hif2a* deficient mice upon inspiratory hypoxia (Figure 4), its expression in the very acute stage after ischemic stroke was comparable to that in WT mice (Figure 7(d)). Glucose transporters (Glut1, Glut3) were up-regulated in the ipsilateral hemisphere of *Hif1a/Hif2a* deficient, but not in WT mice, and *Glut1* expression in KO was significantly higher as compared to WT. Irrespective of genotype, during early acute

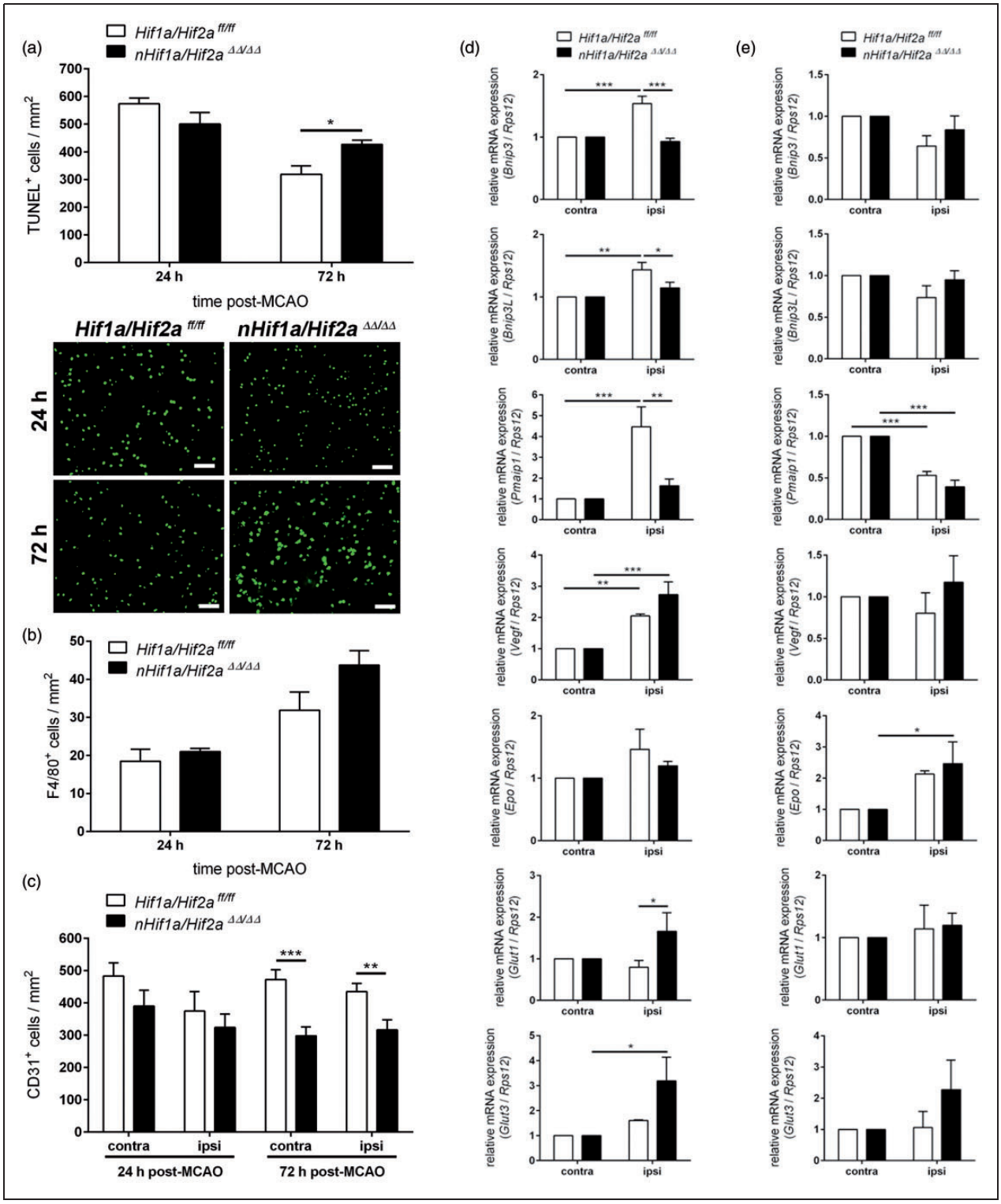

Figure 7. Neuronal deficiency for *Hif1a* and *Hif2a* reduces expression of anti-survival genes in the early acute phase, while it increases apoptosis and decreases angiogenesis at the acute stage of mild ischemic stroke. (a) Mice were subjected to 30 min of MCAO followed by 24 h ($n = 5$–6, per group) and 72 h ($n = 5$, per group) reperfusion, respectively. Brains were removed, coronal cryosections were prepared, and the number of apoptotic cells within the ischemic ipsilateral hemisphere was determined using TUNEL assay. Scale bar = 50 μm. Significant differences determined by unpaired two-tailed t-test are indicated with *($p < 0.05$). (b,c) Mice underwent 30 min of MCAO followed by 24 h ($n = 6$, per group) and 72 h ($n = 6$, per group) reperfusion, respectively. Brains were removed, coronal cryosections were prepared, and the number of CD31-immunoreactive endothelial cells and F4/80-immunoreactive microglia/

(continued)

stage of stroke *Epo* expression was not enhanced within injured brain tissue (Figure 7(d)); 72 h upon ischemic insult during transition from acute toward sub-acute stages, none of the studied HIF-targeted factors were significantly up-regulated in the ipsilateral hemisphere as compared to contralateral control tissue in both *Hif1a*/*Hif2a* deficient and WT animals (Figure 7(e)). The only exception was *Epo* that showed significantly increased transcription in *Hif1a*/*Hif2a* deficient mice and a tendency toward up-regulation in WT (Figure 7(e)). In spite of comparable *Vegf* expression, we determined a significantly reduced density of microvascular endothelial cells in both ipsi- and contralateral hemisphere of *Hif1a*/*Hif2a* deficient mice in the acute phase, while during the early acute stage, the number of endothelial cells was not significantly different between *Hif1a*/*Hif2a* deficient and WT animals that suffered from mild ischemic stroke (Figure 7(c)).

These results support our hypothesis that neuronal HIF deficiency is favorable early after stroke but becomes awkward at later stages.

Discussion

Hypoxia-/ischemia-induced factors play a major role in the pathogenesis of stroke recovery. Many of them are HIF-target genes such as Epo and VEGF both of which are neuroprotective and result in reduced infarct size after cerebral ischemia.[34,35,43,44] However, both factors also have undesired side effects when given as treatment, such as erythrocytosis and vascular leakage leading to brain edema.[34,45] Understanding the in vivo situation where not only a single factor but the whole hypoxia-induced pathway is activated might help to find alternative therapeutic strategies. We recently showed that neuronal inactivation of PHD2 results in up-regulation of HIF-α, Epo, VEGF, glucose transporters, and glycolytic enzymes after cerebral ischemia, thereby leading to smaller infarct size but also reduced edema formation, suggesting that simultaneous activation of several HIF targets could counteract negative side effects of single target genes[27] and might represent the pathophysiological basis for stroke recovery.

Role of HIF-1α and HIF-2α

To further characterize the PHD-HIF-axis in stroke recovery, we generated mice with single or combined neuronal deficiency for HIF-1α and HIF-2α. Our current in vivo findings do not indicate that neuron-specific lack of HIF-1 function alters neuronal cell death upon mild or severe ischemic insult, and may reflect discrepant results in the literature. While Helton et al.[6] reported decreased tissue damage upon cerebral ischemia, Baranova et al.[5] found increased ischemic brain injury due to the loss of HIF-1 in neurons. The observed differences might be due to the use of diverse murine stroke models, i.e. global brain ischemia using transient bilateral occlusion of the common carotid artery[6] or focal ischemia induced by transient MCAO.[5] Although we also applied the intraluminal filament technique to transiently occlude the MCA, our Cre recombinase driver mouse line[26] used to generate neuron-specific gene knockout was different from that described in the previous reports, which might also explain differing observations.

Our current results indicate that HIF-2 may substitute for HIF-1 in *Hif1a* deficient neurons. Indeed, up-regulation of HIF-regulated genes including *Vegf*, *Glut1*, *Glut3*, and *Pmaip1* in response to a short hypoxic episode was unaffected in brain tissue of *Hif1a* deficient mice and comparable to WT mice. Similar results were reported by Helton et al.,[6] when studying overall gene expression through microarray analysis in brain tissue upon hypoxic insult. However, we also found opposed responses. Hypoxic induction of *Epo* in nervous tissue of neuronal *Hif1a* knockout mice was not only not reduced, but even enhanced compared to WT, and *Bnip3* expression was clearly attenuated under hypoxia, implying that *Epo* transcription in neurons is primarily regulated by HIF-2, while *Bnip3* is a HIF-1 target. Similar to our observation, two previous studies showed that transcriptional activation of *Bnip3*, a pro-apoptotic member of the Bcl-2 protein family, in renal cell carcinoma and hepatocellular tumor spheroids is predominantly induced by HIF-1.[46,47] Along the same line, chromatin immunoprecipitation analyses in mice exposed to mild hypoxia demonstrated that *Epo* expression in brain tissue is induced by HIF-2.[48] Moreover, abrogation of HIF-2α but not HIF-1α

Figure 7. Continued
macrophages within non-ischemic contralateral and ischemic ipsilateral hemisphere was determined using immunofluorescence microscopy. (b) Unpaired two-tailed t-test was applied to determine statistical significance. (c) Significant differences determined by two-way ANOVA with Holm-Sidak post-hoc test are indicated with **($p < 0.01$) or ***($p < 0.001$). Mice were subjected to 30 min of MCAO followed by (d) 6 h ($n = 5$, per group) and (e) 72 h ($n = 4$–5, per group) reperfusion, respectively. Gene expression in the contralateral and ipsilateral hemispheres was analyzed by real-time PCR. Values are normalized to *Rps12* mRNA levels and expressed as fold change to contralateral *Hif1a*/*Hif2a*$^{f/f}$ and *nHif1a*/*Hif2a* $^{\Delta\Delta/\Delta\Delta}$, respectively. Significant differences determined by two-way ANOVA with Holm-Sidak post-hoc test are indicated with *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$).

expression in cultured astrocytes led to a drastic decrease of hypoxic *Epo* expression.[49] Accordingly, multiple genetic studies in mice provided evidence that, in the adult, renal and hepatic Epo synthesis is predominantly HIF-2- and not HIF-1-regulated.[38] Interestingly, Baranova et al.[5] also observed enhanced *Epo* expression, which was accompanied with higher HIF-2α protein levels in cerebral tissue of *Hif1a* deficient animals. The phenomenon of increased HIF-2α protein levels in the absence of HIF-1α was also found by others in several cancer cell lines.[47,50] By contrast, we did not find any compensatory up-regulation of *Hif2a* on mRNA or protein level in neuronal *Hif1a* knockout mice, which could have explained increased *Epo* expression. However, on cellular level we cannot exclude a slight up-regulation of HIF-2 in *Hif1a* deficient neurons.

To further study potential redundancy of HIF-1 and HIF-2 for the neuronal adaptive response to hypoxic/ischemic insults, we generated transgenic mice with neuronal deficiency for *Hif2a*. In contrast to 90% overall reduction of hypoxic HIF-1α levels in brains of *Hif1a* deficient mice, indicative of neurons as major CNS source of HIF-1, hypoxic HIF-2α levels in cerebral tissue of *Hif2a* deficient animals were only slightly decreased by approximately 35%, suggesting HIF-2 rather derives from non-neuronal cells such as glial and endothelial cells. Gene expression of classical HIF-target genes in hypoxic brain parenchyma of *Hif2a* knockout mice was not significantly different from that in WT with the exception of *Epo*. Hypoxia-induced up-regulation of *Epo* was abrogated due to neuronal *Hif2a* deficiency, further underlining the importance of HIF-2 for transcriptional activation of the *Epo* gene and the significant neuronal origin of this factor in the CNS. Accordingly, irrespective of severity of the ischemic insult, global brain tissue damage and edema expansion in neuron-specific *Hif2a* deficient mice were unaltered as compared to WT littermates. Overall, we provide evidence that in animals exposed to hypoxic/ischemic conditions neuronal loss of HIF-1 can be compensated by HIF-2, and vice versa.

Tissue protection after completely impairing neuronal HIF function

In order to circumvent potential compensatory mechanisms, we used conditional knockout mice deficient for both *Hif1a* and *Hif2a*. Hypoxic up-regulation of several HIF-regulated factors such as *Epo*, *Glut-1*, and *Pmaip1* was only slightly attenuated in *Hif1/2 a* double deficient mice, which might reflect the contribution of other resident brain cells like astrocytes, endothelial cells, oligodendrocytes, or microglia to total mRNA levels of these genes during the hypoxic adaptive response. However, in comparison to WT mice subjected to short global inspiratory hypoxia, ablation of both HIF-α subunits in neurons almost completely prevented up-regulation of *Vegf* and *Bnip3* in the hypoxic forebrain indicating neurons as substantial cellular source for VEGF and BNIP3, respectively. In accordance with our findings, several previous studies identified neurons as important source for VEGF and BNIP3 expression in brains of rodents and human patients suffering from ischemic stroke.[2,51–53] However, it should be considered that during focal brain ischemia caused by occlusion of a cerebral artery, in contrast to global inspiratory hypoxia, the oxygen availability gradually decreases from the center to the periphery of the ischemic lesion leading to a broad spectrum of HIF activity levels, highest in the center toward lower levels in the periphery. Furthermore, additional lack of supply with glucose and further energy substrates might directly or indirectly affect stability and/or activity of HIFs which could influence the spectrum and strength of HIF-target gene induction. In accordance with these assumptions, in the very acute stage of mild ischemic stroke, neuronal *Hifa* deficiency not only attenuated up-regulation of *Bnip3* but also prevented stroke-induced expression of further HIF-targeted cell death factors such as *Bnip3L* and *Pmaip1*. Moreover, in contrast to global hypoxia, stroke-induced *Vegf* up-regulation was not affected in *Hifa* deficient mice. Our comprehensive histological and functional investigations revealed that in *Hifa* deficient animals subjected to mild ischemic stroke, infarct, and edema size as well as sensorimotor deficit in the early acute (24 h after onset of reperfusion) stage of stroke were clearly reduced in comparison to WT. By contrast, in the acute stage (72 h after onset of reperfusion) this protective effect was completely lost, and global lesion and edema volume were no longer different from WT. Furthermore, sensorimotor dysfunction was now markedly aggravated, suggesting that blockage of the HIF pathway in neurons enhances early acute outcome from ischemic stroke, while it impairs neuronal recovery in the acute and likely sub-acute phase.

One might speculate that diminished up-regulation of anti-survival factors like BNIP3, BNIP3L, and PMAIP1 in neurons in response to an ischemic insult might contribute to diminished early cell death and sensorimotor dysfunction in *Hifa* deficient mice. Accordingly, BNIP3 has been demonstrated to be up-regulated predominantly in neurons after ischemic stroke leading to neuronal cell death by activating excessive mitophagy.[2,54] Furthermore, mice deficient for BNIP3 show significantly reduced neuronal mitophagy and apoptosis following ischemic/hypoxic insult.[54] Similarly, Chen et al.[55,56] demonstrated in a rat model of transient cerebral ischemia that early

inhibition of HIF-1 by application of inhibitors or small interfering RNA attenuated up-regulation of VEGF and BNIP3, reduced the infarct volume and blood–brain barrier (BBB) hyperpermeability, decreased mortality and improved neurological deficits. Moreover, acutely enhanced expression of PMAIP1 and BNIP3L has been shown in a rodent model of ischemic stroke, and suppression of PMAIP1 expression by delivery of antisense oligonucleotides markedly reduced cerebral infarction.[4,57]

In contrast, global *Vegf* expression was not altered within the entire ischemic lesion of *Hif1/2 a* double knockout mice at both early acute and acute stage of mild ischemic stroke. However, we cannot fully exclude that post-ischemic *Vegf* expression is at least focally attenuated in *Hif1a*/*Hif2a* deficient neurons, which might enhance neuronal survival during the early acute phase of ischemic stroke by attenuating post-ischemic BBB dysfunction and vasogenic edema formation. Contrary, in late acute and sub-acute stages, the lack of autocrine/paracrine VEGF as potent antiapoptotic mediator[35,44] might enhance the delayed programmed cell death of compromised neurons located in peri-infarct regions that may contribute to impaired functional outcome. Accordingly, we have shown that in the acute phase after mild ischemic stroke the apoptosis rate was substantially enhanced in *Hif1a*/*Hif2a* deficient mice. Moreover, angiogenesis and neurogenesis, adaptive cellular mechanisms strongly contributing to long-term regeneration from ischemic stroke and known to be potently induced by VEGF,[35,58] might be impaired due to the loss of neuronal VEGF production. Indeed, the capillary density in ischemic brains of *Hif1a*/*Hif2a* deficient mice was markedly reduced in the acute phase, whereas cerebral microvasculature in the early acute stage was not different between knockout and WT animals. In accordance with our findings, Zhang et al.[45]showed in a rat model of focal cerebral embolic ischemia that early post-ischemic (1 h) administration of recombinant human VEGF significantly increased BBB leakage, hemorrhagic transformation, and ischemic lesions, while late (48 h) administration of VEGF enhanced angiogenesis in the ischemic penumbra and significantly improved neurological recovery.[45]

In summary, our results suggest that neuronal HIF-1α and HIF-2α can partially compensate for each other, although specific target genes are differentially regulated after hypoxia/ischemia. We also show that combined loss of neuronal HIF-1α and HIF-2α impairs functional recovery after cerebral ischemia, although it may be beneficial in the early acute phase after stroke, indicating that a timely regulated activation/inhibition of hypoxia-regulated cytoprotective and damaging factors might be of major importance for the functional outcome after stroke. This should be considered when using HIF activators or repressors for therapeutic purposes.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

PB performed the experiments, analyzed data, and contributed to the writing of the manuscript. LL, ASE, and LIB performed experiments. HHM contributed to the experimental design and the writing of the manuscript. RK conducted experimental design of the study, analyzed data, and wrote the manuscript.

Supplementary material

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data

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