Neuron-Specific Prolyl-4-Hydroxylase Domain 2 Knockout Reduces Brain Injury After Transient Cerebral Ischemia

Reiner Kunze, PhD; Wei Zhou, MD; Roland Veltkamp, MD; Ben Wielockx, PhD; Georg Breier, PhD; Hugo H. Marti, MD

**Background and Purpose**—Numerous factors involved in the adaptive response to hypoxia, including erythropoietin and vascular endothelial growth factor are transcriptionally regulated by hypoxia-inducible factors (HIFs). During normoxia, prolyl-4-hydroxylase domain (PHD) proteins hydroxylate HIF-α subunits, resulting in their degradation. We investigated the effect of neuronal deletion of PHD2, the most abundant isoform in brain, for stroke outcome.

**Methods**—We generated neuron-specific Phd2 knockout mice and subjected animals to systemic hypoxia or transient middle cerebral artery occlusion. Infarct volume and cell death were determined by histology. HIF-1α, HIF-2α, and HIF target genes were analyzed by immunoblotting and real-time polymerase chain reaction, respectively.

**Results**—Neuron-specific ablation of Phd2 significantly increased protein stability of HIF-1α and HIF-2α in the forebrain and enhanced expression of the neuroprotective HIF target genes erythropoietin and vascular endothelial growth factor as well as glucose transporter and glycolysis-related enzymes under hypoxic and ischemic conditions. Mice with Phd2-deficient neurons subjected to transient cerebral ischemia exhibited a strong reduction in infarct size, and cell death of hippocampal CA1 neurons located in the peri-infarct region was dramatically reduced in these mice. Vessel density in forebrain subregions, except for caudate–putamen, was not altered in Phd2-deficient animals.

**Conclusions**—Our findings denote that the endogenous adaptive response on hypoxic–ischemic insults in the brain is at least partly dependent on the activity of HIFs and identify PHD2 as the key regulator for the protective hypoxia response. The results suggest that specific inhibition of PHD2 may provide a useful therapeutic strategy to protect brain tissue from ischemic injury. (Stroke. 2012;43:2748-2756.)

Key Words: HIF-1α ▪ hypoxia ▪ neuroprotection ▪ PHD ▪ stroke

Neurological defects caused by cerebral ischemia are primarily due to massive neuronal malfunction and cell death. However, the decline of cellular oxygen level during ischemia also induces various neurotrophic mediators, which protect neurons from ischemic cell death and form part of an endogenous adaptive response aiming to defend and recover brain tissue from ischemic injury.1 Within this neuroprotective response, the switch from aerobic to anaerobic glucose metabolism by upregulating glucose transporters (Glut1, Glut3) and glycolysis-related enzymes such as phosphofructokinase 1, fructose-bisphosphate aldolase, phosphoglycerate kinase 1, pyruvate dehydrogenase kinase 1 (Pdk1), and lactate dehydrogenase is one of the key mechanisms to maintain cellular energy production and thus survival of neurons during ischemia.2,3 Moreover, vascular endothelial growth factor (VEGF) and erythropoietin (Epo) are strongly expressed in the brain during stroke and promote neuronal survival through induction of antiapoptotic pathways.4–7 The expression of these factors is mainly controlled by hypoxia-inducible transcription factors (HIFs), which are heterodimeric proteins composed of an α (1α, 2α, or 3α) and a β subunit.8 Under normoxic conditions, the α subunit is hydroxylated on prolines 402 and 564 leading to binding of the von Hippel-Lindau protein E3 ubiquitin ligase and its subsequent proteasomal degradation. This prolyl hydroxylation is mediated through a family of prolyl-4-hydroxylase domain (PHD) proteins by using molecular oxygen.8 Under hypoxic–ischemic conditions, in which oxygen is limited, PHDs are less active and thus hypohydroxylated HIF-α becomes stabilized resulting in transcription of HIF target genes.8 Three PHD family members have been identified (PHD1-3),8 which show a tissue-specific abundance and different substrate specificity toward the HIF-α.
In the brain of adult mice, PHD2 is the most abundant isoform, and features the highest specificity toward HIF-1α. Previous studies showed that pharmacological inhibition of PHD can reduce acute neuronal injury in animal models of experimental stroke by induction of the HIF-1 pathway. However, currently tested PHD inhibitors such as dimethyl-oxalylglycine (DMOG), deferoxamine mesylate, and 3,4-dihydroxybenzoate do not allow a PHD isoform-selective inactivation and may in addition influence the enzymatic activity of proteins other than PHDs. We used genetically modified mice with neuron-specific ablation of PHD2 and subjected them to transient cerebral ischemia to verify our hypothesis that PHD2 is the key regulator of the endogenous adaptive response on hypoxic–ischemic insults in the brain.

### Materials and Methods

The study was approved by the governmental animal care authorities (Regierungspräsidium Karlsruhe, Germany). All mice (males, 8–10 weeks old) used in the experiments were randomized. Surgeon and investigators were blinded to the mouse genotype. All assessments and analyses were done independently and blinded. Mice were housed and bred in the Interfacultäre Biomedizinische Forschungseinrichtung (IBF), University of Heidelberg.

### Generation of Neuron-Specific Phd2 Knockout Mice

The generation and detailed characterization of Phd2KO mice will be reported elsewhere (G.B., B.W., unpublished data). To construct neuron-specific Phd2 knockout animals, we crossed Phd2KO mice with a transgenic mouse line expressing Cre recombinase under control of the Ca2+/calmodulin-dependent pro-
tein kinase II promoter (CaMKII-Cre^{+/−} kindly provided by Dr Günther Schütz, German Cancer Research Center, Heidelberg, Germany).16 Phd2^{lox/lox} X CaMKII-Cre^{+/−} mice were crossed to Phd2^{lox/lox} and Phd2^{lox/+} mice to obtain homozygous (nPhd2^{+/−}) and heterozygous (nPhd2^{+/−}) neuron-restricted Phd2 knockout mice as well as littermate control wild-type (WT) mice (Phd2^{lox/lox}). CaMKII-Cre^{+/−} mice were bred with the ROSA26 reporter (R26R) mouse line to monitor spatial pattern of Cre activity by X-gal staining as previously described.17,18 Genotyping and analysis of Cre-mediated excision of floxed Phd2 sequence were performed using polymerase chain reaction (online-only Data Supplement Methods).

Middle Cerebral Artery Occlusion and Systemic Hypoxia

Focal cerebral ischemia was induced using an intraluminal technique (online-only Data Supplement Methods). Mice were exposed to normobaric hypoxia at 6% oxygen for 12 hours or kept at 20% oxygen as described.11 Mice were euthanized by decapitation and brains isolated and frozen for subsequent analysis.

Immunohistochemistry

Immunohistochemical techniques were used to detect Cre and PHD2 expression and to visualize CD31-positive blood vessels in brain sections (online-only Data Supplement Methods).

Figure 2. Characterization of neuron-specific Phd2 knockout in Phd2^{lox/lox} X CaMKII-Cre mice. Expression of the PHD isoforms was quantified in the forebrain (F) and cerebellum (CB) of Phd2^{lox/lox}, nPhd2^{+/−}, and nPhd2^{+/−} mice by Western blot (nuclear protein extracts; A) and real-time polymerase chain reaction (B). Gene expression was normalized to Rps12 and is expressed as fold change to Phd2^{lox/lox} for the same gene (B). Significant differences determined by 2-way analysis of variance combined with Bonferroni posttest are indicated with ***P<0.001. N=3 (per group). PHD indicates prolyl-4-hydroxylase domain.

Transferase-Mediated dUTP Nick-End Labeling Assay

Apopotic cells were detected in brain sections using the transferase-mediated dUTP nick-end labeling assay (online-only Data Supplement Methods).

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction was performed as described.11 For primer sequences (MWG Biotech, Ebersberg, Germany), see online-only Data Supplement Table I.

Immunoblotting

Nuclear and cytosolic proteins were extracted from brain tissue and immunoblotting was performed to analyze expression of HIF-1α, HIF-2α (nuclear protein extracts), and PHD1-3 (nuclear/cytosolic protein extracts; online-only Data Supplement Methods).

Statistical Analysis

The primary outcome of the study was infarct volume at 24 hours after middle cerebral artery occlusion. Sample size calculation, based on an expected 50% reduction in infarct size, and a 40% SD with an α and β error of 5%, indicated a sample size of 9 animals per group. However, 2 animals from the WT group had to be excluded from analysis because they did not show a sufficient reduction in
cerebral blood flow (measured by laser-Doppler monitoring) when the filament was inserted. Thus, 7 and 9 animals per group were used for the final analysis. The results are presented as mean/SEM. Unpaired 2-tailed t test or 2-way analysis of variance combined with Bonferroni posttest was applied to determine statistical significance.

Results

Characterization of Neuron-Specific Phd2 Knockout Mice

We crossed CaMKIIα-Cre mice with R26R mice and analyzed sagittal brain sections of adult double-transgenic mice by X-Gal staining (Figure 1A). We found that Cre/loxP recombination, as illustrated by blue staining, was mainly restricted to forebrain structures including the hippocampus, striatum, and cortex, but was also detected in cerebellar Purkinje cells (Figure 1A). Furthermore, immunohistochemical analysis revealed a high frequency of Cre recombinase-positive neurons in the hippocampus of adult nPhd2 mice (Figure 1B). In contrast, no staining was observed in brain sections of R26R and Phd2flox/flox mice, respectively (Figure 1A–B). Consistently, Cre-mediated excision of floxed Phd2 exons 2 and 3 was demonstrated on the genomic DNA level in the forebrain and also faintly in the cerebellum of nPhd2 mice and to a lesser extent in nPhd2 mice, whereas in other tissues, the Phd2 alleles were unaltered (data not shown). PHD2 protein was markedly decreased in the hippocampus and cortex of nPhd2 mice (Figure 1C). More-
normoxic conditions (Figure 3A). Although cerebral hypoxia increased the protein stability of both HIF-α isoforms in WT and nPhd2ΔΔ mice, HIF-1α but not HIF-2α abundance was higher in the forebrain of nPhd2ΔΔ mice as compared with WT (Figure 3A). In accordance, mRNA expression of the HIF target genes Vegf, Epo, Phd3 and factors involved in glucose metabolism (Glut1, Glut3, phosphofructokinase 1, phosphoglycerate kinase 1, Pdk1, lactate dehydrogenase) was significantly increased in the forebrain of nPhd2ΔΔ mice as compared with WT under hypoxic conditions (Figure 3B). Of note, increased Phd3 mRNA did not result in increased protein levels (Figure 3A). By contrast, normoxic expression of HIF target genes with the exception of Epo was not different between WT and nPhd2ΔΔ animals. In comparison to WT, Epo transcription was 5-fold increased in the forebrain of normoxic nPhd2ΔΔ mice (Figure 3B).

**Neuron-Specific Phd2 Inactivation Reduces Brain Injury After Transient Cerebral Ischemia**

We analyzed if forebrain-restricted inactivation of Phd2 improves outcome from acute ischemic stroke. As shown in Figure 4A, infarct size of nPhd2ΔΔ mice was significantly reduced by >50% as compared with WT mice. In addition, neuronal cell death/apoptosis was dramatically reduced in peri-infarct regions as determined by a decreased number of transferase-mediated dUTP nick-end labeling-positive hippocampal CA1 neurons (Figure 4B). nPhd2ΔΔ mice showed a 2- to 3-fold increased HIF-1α protein expression in both ischemic ipsilateral and nonischemic contralateral hemisphere as compared with WT mice (Figure 5A). HIF-1α protein levels 24 hours after ischemia were similar in both hemispheres of WT and nPhd2ΔΔ mice, respectively (Figure 5A), which does not seem remarkable because cerebral induction of HIF-1α protein by transient middle cerebral artery occlusion in mice usually peaks 4 to 8 hours after ischemia and then declines to basal levels. The expression of the HIF-regulated genes Epo, Vegf, Phd3, Glut1, phosphofructokinase 1, phosphoglycerate kinase 1, and lactate dehydrogenase was significantly increased in the ipsilateral hemisphere of nPhd2ΔΔ mice as compared with corresponding brain tissue in WT animals (Figure 5B; online-only Data Supplement Figure II). However, enhanced Phd3 mRNA expression did not result in increased PHD3 protein in ischemic nPhd2ΔΔ brain (Figure 5A).

**Forebrain-Restricted Phd2 Knockout Mice Show Increased Vessel Density in the Caudate–Putamen**

Although neuronal Phd2 deletion had no effect on basal VEGF expression, the stronger hypoxic inducibility (Figure 3B) prompted us to investigate whether neuronal Phd2 inactivation has consequences for capillary density in the brain, and we quantified vascular density in different forebrain regions of nPhd2ΔΔ mice. Interestingly, nPhd2ΔΔ animals showed a 40% increased vessel density in caudate–putamen, whereas there was no significant difference in any other brain region analyzed (Figure 6).

**Discussion**

To the best of our knowledge, this is the first report demonstrating that neuron-restricted deletion of a single member of the PHD family is sufficient to activate the hypoxic adaptive response and confer neuroprotection during cerebral ischemia.
PHD2 Is a Key Regulator of the Hypoxia-Adaptive Response to Confer Neuroprotection

Our results that HIF-1α and to a lesser extent HIF-2α protein abundance is strongly enhanced in the forebrain of nPhd2/flox/flox mice suggest that neither PHD1 nor PHD3 can compensate for the loss of PHD2 function and confirm that PHD2 plays a key role in regulating steady-state levels of HIF-1α under normoxic conditions. Moreover, our results indicate that improved outcome from acute ischemic stroke in nPhd2/flox/flox mice is at least partly due to induction of the HIF pathway. Accordingly, Hölscher et al19 reported that cell-type specific Phd2 ablation in cardiomyocytes causes HIF-1α stabilization in murine hearts and protects from acute myocardial ischemic injury. Similarly, isolated hearts of Phd2 hypomorphic mice exhibit increased HIF-1α and HIF-2α protein amounts and were protected against ischemic–reperfusion injury,20 and cardiac repression of Phd2 but not Phd1 or Phd3 by RNA interference technique resulted in activation of HIF-1α and attenuated myocardial infarct.21 The outstanding role of PHD2 as a key regulator of adaptive responses to hypoxia–ischemia is further supported by a previous study showing that Phd2 null mice die during embryonic development due to severe placental and heart defects, whereas Phd1 and Phd3 null mice are viable.22 Further evidence for a key role of PHD2 in the central nervous system comes from our recent finding that Phd2 is the most abundant family member in the brain.11 Neuron-specific deletion of Phd2 in the forebrain resulted in >90% reduction in protein level in brain hemispheres (Figure 2). Taking into account that glial and endothelial cells outnumber neuronal cells by at least 10-fold, our results (Figure 1D) suggest that neuronal cells express considerably more PHD2 in comparison with other cell types. Accordingly, cell-type specific PHD expression was also demonstrated in other organs, for example, rodent kidney, in which tubular cells expressed all 3 PHDs, whereas in peritubular interstitial cells and glomerular podocytes, only PHD1 and PHD3 were detectable.23 Although our results (Figure 1) and data from the literature indicate that the CaMKII promoter is neuron-specific,13,24 we cannot rule out that the promoter shows some minor activity in nonneuronal cell types with subsequent Cre recombinase-mediated deletion of Phd2 in those cells.

Involvement of the HIF Pathway

PHD inhibition has been previously suggested to be an attractive target to confer neuroprotection.12 Indeed, 2 recent reports suggest that treatment with the PHD inhibitor DMOG results in neuroprotection after cerebral ischemia.14,15 However, DMOG as a 2-oxoglutarate analog is likely to interfere also with 2-oxogluarate-dependent enzymes other than PHDs such as collagen prolyl-hydroxylase and citric acid cycle-derived α-ketoglutarate dehydrogenase.25,26 Furthermore, DMOG nonselectively inhibits all PHD family members, not allowing to discriminate the importance of single members. By contrast, the cell-specific ablation of Phd2 presented here allowed us to directly allocate the central role of this factor for neuroprotection. PHD2 is best known for its role as a cellular oxygen sensor by regulation of HIF transcription...
subunits was strongly increased in the forebrain of these mice. In addition to prolyl hydroxylation by the PHD isoenzymes, HIF-1α is hydroxylated on an asparaginyl residue in an oxygen-dependent manner through factor-inhibiting HIF-1.33 Factor-inhibiting HIF-1-mediated hydroxylation does not affect the protein stability of HIF-1α but blocks its interaction with the transcriptional coactivators CBP/p300 required for full transcriptional activity of HIF-1.33 Thus, one can argue although PHD1 and PHD3 cannot impede HIF-1α stabilization in Phd2-deficient brain tissue under normoxia, factor-inhibiting HIF-1 would prevent the transcriptional activity of the stabilized HIF-1. However, under hypoxic conditions, factor-inhibiting HIF-1, like the PHDs, is inactive, enabling expression of numerous HIF target genes.33 Furthermore, factor-inhibiting HIF-1 was reported to act less active on HIF-2α than on HIF-1α, conferring increased HIF-2 transcriptional activity under normoxia.34,35 Because Epo expression in the brain is controlled by HIF-2 rather than HIF-1,36,37 this, together with our finding that HIF-2α protein is stabilized in healthy nPhd2Δ/Δ mice, strongly indicates that Epo upregulation in Phd2-deficient brain in normoxia is at least partially mediated in a HIF-2-dependent manner.

The strong upregulation of Epo in our mouse model indicates its central role as a neurotrophic and neuroprotective factor in the central nervous system.7 Further evidence for Epo as a main factor mediating hypoxic adaptation comes from preconditioning experiments in which a protective effect of initial hypoxic exposure on subsequent cerebral ischemia was Epo-dependent.38 Nevertheless, although initial studies favored a protective role of Epo also in clinical studies,39 a later multicenter Phase II/III trial did not support this notion.40 Thus, additional factors such as VEGF might be needed to improve outcome after stroke.

Although healthy adult nPhd2Δ/Δ mice did not show elevated levels of Vegf, we found a significant increased vascular density in the caudate–putamen. Thus, VEGF levels might have been increased during earlier periods of life. Alternatively, increased vessel density might be the result of Epo action, because Epo has a mitogenic effect on brain capillary endothelial cells.41 In nPhd2Δ/Δ mice, infarct size was reduced both in the cortex and striatum. Taking into account the lack of increased capillary density in cortex, our results suggest that angiogenesis is not making a primary contribution to the reduction in infarct size. This notion is supported by our earlier findings in mice with brain-specific overexpression of VEGF. Despite increased vascular density in those mice, the presence of a hemodynamic steal phenomenon strongly indicated that the major effect of VEGF in this setting was direct neuroprotection rather than angiogenesis.42

On the other hand, VEGF has a strong permeability inducing effect,43 and indeed, VEGF transgenic mice showed increased vascular leakage after ischemia.5 However, in nPhd2Δ/Δ mice, no change in edema formation as compared with WT mice was noted (10.1 ± 3.5 versus 12.7 ± 4.1 mm3) despite higher VEGF levels during ischemia. These results suggest that coordinated activation of the whole hypoxic pathway, including Epo, can impede permeability effects of VEGF. Indeed, Epo has been shown to protect against VEGF-mediated vascular leakage.43 Thus, although initially
identified as permeability inducing and angiogenic factor, the major effect of VEGF upregulation during ischemia in the brain of our nPhd2Δ/Δ mice is likely to be neurotrophic and neuroprotective, as has been suggested for many neurological disorders.42

In addition, Phd2 inactivation resulted in upregulation of glycolytic pathways, which likely contribute to neuronal survival. Reduced blood flow to the brain during ischemic stroke causes insufficient oxygen and glucose supply required for neuronal energy production. Acute protection of Phd2-deficient neurons against cerebral ischemia may at least in part be facilitated by increased glucose uptake and adenosine triphosphate production through anaerobic glycolysis. In accordance, Aragones et al44 showed that loss of Phd1 provides protection of myofibers against lethal ischemia by reprogramming glucose metabolism from oxidative to more anaerobic adenosine triphosphate production.

Finally, we found no hypoxia–ischemia inducibility for Phd2 and Phd3 at least on the protein level in brains of both WT and nPhd2Δ/Δ mice (Figures 3 and 5) in contrast to other reports analyzing liver, heart, and skeletal muscle tissues.9,44 However, our findings are in agreement with previous reports demonstrating that neither Phd2 nor Phd3 expression is significantly increased in brain tissue after hypoxia.9,11,45

PHD Inhibition as a Therapeutic Option for Treatment of Stroke

Our findings denote that inhibition of PHD2 may provide a useful therapeutic strategy to protect brain tissue from ischemic injury. Previous Phase I clinical trials demonstrated that oral administration of the PHD inhibitor FG-2216 (FibroGen, Inc) significantly increased Epo production in anemic patients on hemodialysis with chronic kidney disease.46 Moreover, a related PHD inhibitor (FG-4497) enhanced proliferation, survival, and dopaminergic differentiation of human fetal mesencephalic neural progenitor cells in vitro, whereas other HIF-stabilizing agents such as deferoxamine mesylate and DMOG were toxic.47 However, because these drugs are 2-oxoglutarate analogs like DMOG, development of more specific and isoform-selective PHD inhibitors seems indispensable.

In summary, our data demonstrate that neuronal inactivation of a single PHD family member, PHD2, is sufficient to activate hypoxic adaptive pathways with upregulation of a whole battery of factors, among them Epo and VEGF, and to result in significant neuroprotection during cerebral ischemia.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

Supplemental Methods

Genotyping and analysis of Cre-mediated excision of floxed Phd2 sequence

Genotyping was performed using PCR. Phd2 floxed and WT alleles were detected using the following primers: Phd2 fwd 5´-CTCACTGACCTACGCGGTGT-3´ and Phd2 rev 5´-CGCATTTCCATCTCCATTT-3´. Transgenic mice expressing Cre recombinase and having the ROSA26 locus were identified using following primers: Cam-cre1 5´-GGTTCTCCGTTCACACTAGGA-3´, Cam-cre2 5´-CTGCATGCAAGGGCAGCTCT-3´ and Cam-cre3 5´-GCTTGCAGGTACAGGAGGTAGT-3´; Rosa26 fwd 5´-TTACCCGTAGGTAGTCAGGCA-3´ and Rosa26 rev 5´-TTACGATGCGCCCATCTACAC-3´. Cre-mediated excision of floxed Phd2 exons 2 and 3 was determined by PCR with the following primers: Phd2 fwd 5´-CTCACTGACCTACGCGGTGT-3´ and Phd2 rev 5´-GGCAGTGATAACAGGTGCAA-3´.

Middle cerebral artery occlusion (MCAO)

Mice were anaesthetized by a mixture containing 4 % halothane, 70 % N2O, and remainder O2, and were maintained by reducing the halothane concentration to 1.0-1.5 %. Body temperature was maintained at 37 °C using a temperature controlled heating pad. A laser-Doppler flowmetry (LDF) probe (Perimed, USA) was positioned 1.5 mm posterior and 3 mm lateral from bregma. A 7.0 silicon rubber-coated monofilament (Doccol Corporation, Redlands, USA) was introduced in the internal carotid artery and pushed toward the MCA until a drop in regional cerebral blood flow (rCBF) below 30 % from baseline was documented by LDF. After 60 min occlusion, reperfusion was allowed for 24 h. For sham surgery, preparation of the common carotid artery was identical, the filament was inserted to occlude the MCA, but was withdrawn immediately to allow prompt reperfusion. During reperfusion mice were kept at 30 °C to prevent hypothermia caused by cerebral ischemia. Subsequently, animals were killed by decapitation, and brains were removed for histologic and biochemical analysis. Brains were embedded into Tissue-Tek (Sakura Finetek, Staufen, Germany) or shock frozen in liquid nitrogen. From each brain, 24 coronal cryosections (10 µm thick each; 0.4 mm apart) were prepared and submitted to cresyl violet staining for quantification of the infarct size and edema size as previously described.1,2

Immunohistochemistry

Sagittal cryosections (10 µm in thickness) were prepared and fixed with zinc-based fixative for 15 min, and incubated for 1 h in blocking buffer containing 5 % goat serum (Dianova, Hamburg, Germany) and 0.1 % Tween-20 in PBS (PBS-T). Then, slices were incubated overnight at 4 °C with an antibody against Cre recombinase (0.05 %; kindly provided by Dr. Günther Schütz, German Cancer Research Center, Heidelberg), followed by incubation with a goat anti-rabbit biotinylated secondary antibody (0.25 %; Vector Labs, Peterborough, UK) for 30 min. Immunoreactivity was visualized by the avidin-biotin complex technique (Vector Labs) in combination with a colorimetric detection method (3,3´-diaminobenzidine) and nuclei were counterstained with hematoxylin.

Sagittal cryosections were fixed with ice-cold methanol for 10 min, and incubated for 30 min in blocking buffer containing 5 % goat serum (Dianova) and PBS-T. Subsequently, slices were incubated overnight at 4 °C with antibodies against PHD2 (B.W.; 0.2 %; custom-made polyclonal rabbit anti-mouse antibody against a C-terminal peptide
(EKGVRVELKPNSVSKDV) and purified via a peptide sulfoLink immobilization column (Pierce, ThermoScientific, IL) and neuronal nuclei (NeuN; 0.2%; MAB377, Millipore, Schwalbach, Germany), followed by incubation with a goat anti-rabbit Cy3- and goat anti-mouse Cy2-conjugated secondary antibody (1%; Dianova), respectively for 1 h. All antibodies were diluted in LowCross-Buffer (Candor, Wangen, Germany). Slices were incubated for 10 min with 0.02 % DAPI (Invitrogen, Darmstadt, Germany) in PBS to stain nuclei. Stained sections on glass slides were then embedded in fluoroshield mounting medium (Sigma-Aldrich, Steinheim, Germany). The staining was visualized using an Olympus BX-50 microscope, and digital images were acquired with a Leica DC 500. The fluorescence signals were detected with the following optics: Cy2: excitation 470-490 nm, emission 510 nm; Cy3: excitation 530-550 nm, emission 590 nm; DAPI: excitation 360-370 nm, emission 420-460 nm.

For CD31 staining, coronal cryosections (10 µm in thickness) were fixed with zinc-based fixative for 30 min and incubated for 30 min in blocking buffer consisting of 10 % rabbit serum (Dianova) in PBS-T. Then, slices were incubated overnight at 4 °C with an antibody against CD31 (2%; 553370, BD, Heidelberg, Germany), followed by incubation with a rabbit anti-rat Cy3-conjugated secondary antibody (0.25%; Dianova) for 1 h. All antibodies were diluted in LowCross-Buffer (Candor). Slices were incubated for 10 min with 0.02 % DAPI (Invitrogen) in PBS to stain nuclei. Stained sections on glass slides were then embedded in fluoroshield mounting medium (Sigma-Aldrich). Whole-brain sections were imaged using a Zeiss Axiovert 200 M fluorescence microscope (objective, 25x) with an attached CCD camera. The fluorescence signals were detected with the following optics: Cy3: excitation 530-550 nm, emission 590 nm; DAPI: excitation 360-370 nm, emission 420-460 nm. By using the TissueQuest software (TissueGnostics, Vienna, Austria) all images were processed and stitched together to generate a single image of the entire brain slice. Five brain sections (from Bregma 1.94 mm to Bregma -2.18 mm) per animal were used to quantify the number of CD31-immunopositive blood vessels in cortex, forceps minor, corpus callosum, caudate-putamen and dentate gyrus, respectively.

**TUNEL-Assay**

Coronal cryosections (10 µm in thickness) were fixed with 3 % paraformaldehyde for 15 min and incubated for 10 min in 0.1 % NaBH₄ in PBS and for 1 min on ice in 0.1 % Triton X-100 in PBS. Extensively washing with PBS and distilled water was followed by overnight incubation at 37 °C in TUNEL reaction mixture (Roche, Mannheim, Germany) containing 10 U/µl terminal deoxynucleotidyl transferase and 300 µM FITC-conjugated dUTP. Subsequently, slices were incubated for 15 min with 0.02% DAPI (Invitrogen) in PBS to stain nuclei. Stained sections on glass slides were then embedded in fluoroshield (Sigma-Aldrich). The staining was visualized using an Olympus BX-50 microscope, and digital images were acquired with a Leica DC 500. The fluorescence signals were detected with the following optics: FITC: excitation 470-490 nm, emission 510 nm; DAPI: excitation 360-370 nm, emission 420-460 nm.

**Immunoblotting**

Brain tissue was homogenized in 750 µl of buffer A containing 10 mM Hapes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 % protease inhibitor cocktail, 1 % Nonidet P40, 2 mM Na₃VO₄ (all from Sigma-Aldrich). After 15 min incubation at 4 °C, samples were centrifuged at 850 g for 10 min. Pellets were resuspended in 1 ml of
buffer A, incubated on ice for 15 min, then centrifuged at 14,000 g for 1 min. Supernatants (cytosolic proteins) from both centrifugation steps were pooled and snap-frozen in liquid nitrogen. Pellets were resuspended in buffer C containing 10 mM Hepes (pH 7.9), 0.4 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 % protease inhibitor cocktail, 2 mM Na3VO4 and incubated on ice for 30 min with gentle shaking. After a final centrifugation at 14,000 g for 10 min, supernatants (nuclear proteins) were snap-frozen in liquid nitrogen. Proteins (25-50 µg) were run on a SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. The TATA binding protein (TBP) and β-actin were used as loading control. The membrane was incubated with primary antibodies against β-actin (0.2 %; A2066, Sigma-Aldrich), HIF-1α (0.2 %; NB100-449, Novus Biologicals, Cambridge, UK), HIF-2α (0.2 %; AF2997, R&D Systems, Wiesbaden, Germany), PHD1 (0.2 %; NB100-310, Novus Biologicals), PHD2 (0.1 %; NB100-2219, Novus Biologicals), PHD3 (0.1 %; NB100-303, Novus Biologicals) or TBP (0.1 %; ab818, Abcam, Cambridge, UK). All antibodies were diluted in 5% non-fat dry milk. After rinsing in tris-buffered saline, blots were incubated with a corresponding horseradish peroxidase-conjugated secondary antibody (1:5000; Thermo Fisher Scientific, Bonn, Germany). ECL Western blot detection reagents (GE Healthcare, Freiburg, Germany) were used for protein detection.

Supplemental Tables

Supplementary Table 1. Primer pairs used for real-time PCR.

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<th>Reverse primer sequence (5´-3´)</th>
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Supplemental Figures and Figure Legends

**Supplementary Figure 1.** Characterization of neuron-specific *Phd2* knockout in *Phd2*\textsuperscript{flox/flox} X *CaMKIIα-Cre* mice. Expression of the PHD isoforms was quantified in cytosolic protein extracts from forebrain (F) and cerebellum (CB) of *Phd2*\textsuperscript{flox/flox}, *nPhd2*\textsuperscript{+/Δ} and *nPhd2*\textsuperscript{Δ/Δ} mice by Western blot. Significant differences determined by two-way ANOVA combined with Bonferroni post-test are indicated with ** (\(p < 0.01\)) or *** (\(p < 0.001\)). \(N = 3\) (per group).

**Supplementary Figure 2.** Neuron-specific *Phd2* inhibition promotes Vegf and Epo transcription in mice undergoing transient cerebral ischemia. Mice were subjected to sham surgery or MCAO for 60 min followed by 24 h reperfusion. Then, brains were isolated, RNA was prepared from ipsilateral hemispheres of *Phd2*\textsuperscript{flox/flox} and *nPhd2*\textsuperscript{Δ/Δ} mice, and Vegf (A) and Epo (B) expression was analyzed by real-time PCR. Values are normalized to *Rps12* and expressed as fold change to sham-operated *Phd2*\textsuperscript{flox/flox} mice. Significant differences determined by two-way ANOVA combined with Bonferroni post-test are indicated with ** (\(p < 0.01\)) or *** (\(p < 0.001\)). \(N = 4\) (per group).
Supplemental References

