Deletion or Inhibition of the Oxygen Sensor PHD1 Protects against Ischemic Stroke via Reprogramming of Neuronal Metabolism

Graphical Abstract

Highlights
- Genetic loss of PHD1 provides substantial protection against brain ischemic injury
- PHD1 loss reprograms glucose metabolism without vascular changes
- Increased oxPPP flux, at the expense of glycolysis, enhances redox balance in neurons
- Intracerebroventricular delivery of anti-PHD1 oligos protects against stroke

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In Brief
How neuronal metabolism can be targeted to promote neuroprotection remains poorly understood. Quaegebeur et al. show that loss or inhibition of the oxygen sensor PHD1 protects against brain ischemia by shunting glucose into the oxidative pentose phosphate pathway, thereby improving oxygen radical detoxification and securing redox homeostasis.

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Deletion or Inhibition of the Oxygen Sensor PHD1 Protects against Ischemic Stroke via Reprogramming of Neuronal Metabolism

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SUMMARY

The oxygen-sensing prolyl hydroxylase domain proteins (PHDs) regulate cellular metabolism, but their role in neuronal metabolism during stroke is unknown. Here we report that PHD1 deficiency provides neuroprotection in a murine model of permanent brain ischemia. This was not due to an increased collateral vessel network. Instead, PHD1−/− neurons were protected against oxygen-nutrient deprivation by reprogramming glucose metabolism. Indeed, PHD1−/− neurons enhanced glucose flux through the oxidative pentose phosphate pathway by diverting glucose away from glycolysis. As a result, PHD1−/− neurons increased their redox buffering capacity to scavenge oxygen radicals in ischemia. Intracerebroventricular injection of PHD1-antisense oligonucleotides reduced the cerebral infarct size and neurological deficits following stroke. These data identify PHD1 as a regulator of neuronal metabolism and a potential therapeutic target in ischemic stroke.

INTRODUCTION

The brain relies primarily on oxidative glucose metabolism (Mergenthaler et al., 2013). However, it remains poorly understood how neurons adapt their metabolism upon ischemic neuronal injury. There is even debate about the metabolism of neurons in baseline conditions, and it remains largely unknown if neuronal metabolism is a target to promote neuroprotection. This is nonetheless an important question, as ischemic stroke is the 4th leading cause of death and a common reason of severe disability. Reperfusion and neuroprotection are two therapeutic strategies aiming to rescue the ischemic penumbra. This region, surrounding a core of necrotic tissue, suffers moderate blood flow reduction and remains viable during a limited period before a failure of energy and redox homeostasis causes neuronal death. Despite progress in preclinical studies, there are no clinically approved neuroprotective treatments.

Prolyl hydroxylase domain proteins (PHD1–3) are master regulators of the response to hypoxia (Quaegebeur and Carmeliet, 2010; Wong et al., 2013). As their hydroxylation of target proteins is oxygen dependent, PHDs act as oxygen sensors. The transcription factors hypoxia-inducible factor (HIF)-1α and HIF-2α are PHD targets, yet other targets exist...
(Wong et al., 2013). When oxygen is present, PHD-mediated hydroxylation targets proteins for proteasomal degradation. Thus, when oxygen levels drop and PHDs lose their activity, HIFs and other target proteins accumulate (Wong et al., 2013).

Little is known about the functions of PHDs in the brain. PHDs instruct metabolic adaptations, but this has been primarily studied in cell types other than neurons. Through stabilization of HIFs, PHD inhibition induces a shift from oxidative to glycolytic metabolism (Aragones et al., 2009). For instance, loss of PHD1 makes the muscle and liver ischemia tolerant by reducing their mitochondrial respiration (Aragones et al., 2008; Schneider et al., 2009). However, it is unknown if PHDs similarly control neuronal metabolism and if HIFs are their primary target. Here, we examined the role of PHD1 in brain ischemia and focused on its role in controlling neuronal metabolism.

RESULTS

PHD1−/− Mice Are Protected against Brain Ischemia

To assess if PHDs regulate brain ischemia, we performed a permanent middle cerebral artery occlusion (pMCAO) in PHD1−/− and PHD3−/− mice and in neural-specific PHD2-deficient mice (PHD2NKO mice). Staining with the vital dye 2,3,5-triphenyltetrazolium chloride (TTC) revealed that the ischemic injury was largely attenuated in PHD1−/− mice (71% reduction in infarct size; Figures 1A–1C) but not in PHD2NKO and PHD3−/− mice (Figures 1D–1I). PHD1−/− mice on a 129S6 background (Figures S1A–S1C) were similarly protected (Figures 1A–1C).

Use of the adhesive tape removal test to quantify functional deficits after pMCAO showed that a delay in time to sense and remove the tape on the affected paw (J) (n = 7 to 8, p < 0.05, repeated-measure ANOVA). Average time mice needed to remove the tape (K) (n = 7 to 8, p < 0.05, repeated-measure ANOVA). All quantitative data are mean ± SEM. *p < 0.05. See also Figure S1.

PHD1 Deficiency Does Not Cause Vascular Changes in the Brain

We investigated a possible vascular mechanism underlying the ischemic protection in PHD1−/− mice, as PHDs regulate vessel growth and perfusion (Quaegebeur and Carmeliet, 2010). Use of the adhesive tape removal test to quantify functional deficits after pMCAO showed that a delay in time to sense and remove the tape on the affected paw was nearly prevented in PHD1−/− mice (Figures 1D–1I). PHD1−/− mice on a 129S6 background (Figures S1A–S1C) were similarly protected (Figures 1A–1C).
PHD1 Deficiency Protects against Oxygen-Nutrient Deprivation

We then hypothesized that PHD1 deficiency might confer neuroprotection by modulating intrinsic neuronal properties and therefore exposed 7 days in vitro (DIV) cortical neurons to 2 hr of oxygen-nutrient deprivation, after which they were re-exposed to ambient air and nutrient-rich medium (Meloni et al., 2011). After 24 hr, neuronal cell death (measured by LDH release) was reduced in PHD1−/− neurons (Figure 2O).

Transcript and protein levels of PHD2 and PHD3 were not-compensatorily upregulated in PHD1−/− neurons (Figures S2D and S2E). Also, PHD2 and PHD3 silencing in WT neurons did not confer protection against oxygen-nutrient deprivation (Figure 2P), all indicating a PHD1-specific effect. The PHD1−/− neuroprotective effect was also not attributable to structural differences in PHD1−/− embryos or different PHD1−/− neuronal culture features (Figures S2F–S2K). Also, mRNA levels of neurotrophic factors and their receptors were not affected by PHD1 deficiency (Table S1).

PHD1 Deficiency Reprograms Central Carbon Metabolism

In muscle, PHD1 deficiency protects against ischemia by lowering respiration and the production of reactive oxygen species (ROS) in the mitochondria via a shift to glycolysis (Aragonés et al., 2008). Surprisingly, however, glycolysis, glucose consumption and glucose oxidation were reduced in PHD1−/− neurons in baseline conditions (Figures 3A–3C). The change in glucose oxidation was not due to a decrease in the number of mitochondria (gDNA/mtDNA ratio: 1.595 ± 0.065 in WT versus 1.565 ± 0.032 in PHD1−/− neurons; n = 3; p = NS) or mitochondrial density (TOMM20 staining) (Figures S3A–S3E). PHD1 deficiency did also not alter the mitochondrial oxygen consumption (OCR) (Figures 3D, 3E, and S3F). As these results (reduced glucose oxidation but similar oxygen consumption in PHD1−/− neurons) were unexpected, we sought to explain this apparent paradox. We hypothesized that oxidation of alternative substrates might be enhanced. Whereas lactate oxidation was unaffected (pmol lactate/hr/10^6 cells: 10,510 ± 3,120 for WT versus 10,440 ± 2,660 for PHD1−/− neurons; n = 3; p = NS), glutamine oxidation was enhanced in PHD1−/− neurons (Figure 3F).

These data suggest that the decrease in ATP production due to the reduction of glucose oxidation was compensated, at least in part, by enhanced production of ATP from increased glutamine oxidation. As a result, PHD1 deficiency did not alter the energy charge ([ATP] + ½ [ADP]) / ([ATP] + [ADP] + [AMP]); 0.947 ± 0.005 for WT versus 0.942 ± 0.005 for PHD1−/− neurons; n = 6; p = NS) or the levels of ATP, ADP, and AMP (Figure S3G). Also, dendritic branching (relying on mitochondrial ATP production) (Oruganty-Das et al., 2012) and other energy-consuming processes (Na+/K+ pump activity, RNA and protein synthesis) were not altered in PHD1−/− neurons (Figures S3H–S3I), implying that PHD1 deficiency in neurons did not induce general energy hypometabolism. Overall, in contrast to PHD1−/− muscle, PHD1−/− neurons did not show a shift from oxidative to glycolytic metabolism, yet maintained mitochondrial metabolism and energy homeostasis, and lowered their glycolytic flux. Notably, the reduced glycolysis was not due to transcriptional changes in glycolytic genes (Table S2).

PHD1−/− Neurons Enhance the oxPPP Flux

Glycolysis in neurons is kept at a low rate to enable flux of glucose into the oxidative pentose phosphate pathway (oxPPP), in which glucose oxidation converts [NADPH^+] to [NADP^+]. The latter is of vital importance as a reducing equivalent to replenish the pool of reduced glutathione (GSH) from oxidized glutathione (GSSG) to maintain redox homeostasis in neurons (Herrero-Mendez et al., 2009). Given this reciprocal regulation of glycolysis and oxPPP, we hypothesized that the reduced glycolysis was part of a glucose rerouting toward the oxPPP. Indeed, PHD1−/− neurons showed a 2.8-fold induction in oxPPP flux levels (Figure 4A), or, when corrected for glycolytic rate (see Experimental Procedures), a 8.7-fold relative increase in oxPPP (n = 4). Thus, in the absence of PHD1, more glucose is redirected from glycolysis toward the oxPPP.

Molecular Regulation of the oxPPP by PHD1 Deficiency

The increased oxPPP was not due to an increased “passive” shunting of accumulating glycolytic intermediates to the oxPPP (Figures 3B and S4A). Also, we did not detect genotypic differences in mRNA levels of glucose-6-phosphate dehydrogenase (G6PD), a rate-limiting enzyme of the oxPPP (Figure S4B), or in G6PD activity (nmol NADH/min/mg protein: 0.433 ± 0.109 for WT versus 0.432 ± 0.109 for PHD1−/−; n = 4; p = NS).

TP53-inducible glycolysis and apoptosis regulator (TIGAR) acts as a putative regulator of the glycolysis/oxPPP crosstalk by redirecting glucose away from glycolysis into the oxPPP (Bensaad et al., 2006), but its function in neurons is poorly characterized. TIGAR transcript levels were 2.2-fold higher in PHD1−/− than WT neurons (Figure 4B), whereas silencing of PHD2 or PHD3 did not affect TIGAR transcript levels (mRNA copies per 10^6 copies β-actin: 0.10 ± 0.01 in ctrl WT, 0.11 ± 0.02 in PHD2KD and 0.11 ± 0.01 in PHD3KD neurons, p = NS). TIGAR also functionally regulated the oxPPP flux, since overexpression of TIGAR in WT neurons increased oxPPP levels (Figure S4C), while silencing of TIGAR in PHD1−/− neurons reduced the oxPPP flux (Figure S4D). Thus, PHD1 deficiency increased the oxPPP flux by regulating TIGAR expression.

PHD1 Deficiency Improves the ROS Scavenging Capacity

The oxPPP generates NADPH, which is used as a reducing equivalent by glutathione reductase to regenerate GSH (Stanton, 228–291, February 9, 2016 ©2016 Elsevier Inc.)
PHD1 Deficiency Maintains a Residual oxPPP flux in Hypoxia

For the oxPPP to provide redox equivalents for neuroprotection during oxygen deprivation (as during stroke), it should remain operational during hypoxia in PHD1−/− neurons. Like in non-neuronal cell types (Aragonés et al., 2009), hypoxia increased the glycolytic flux in neurons, yet glycolysis remained lower in hypoxic PHD1−/− than in WT neurons (Figure 4E). Hypoxia almost nullified the oxPPP flux in WT neurons, while PHD1−/− neurons were still able to maintain a residual oxPPP flux, nearly in the range of normoxic WT neurons (Figure 4F). Notably, the genotypic differences in glucose and glutamine oxidation were maintained in hypoxia as well (Figures S4J and S4K). Thus, by reprogramming glucose metabolism and maintaining a residual oxPPP flux, PHD1 deficiency ensured a low but vital residual level of redox homeostasis, especially during ischemia. Indeed, measurements of oxidized (GSSG) and reduced (GSH) glutathione after 2 hr of oxygen-nutrient deprivation revealed that the glutathione redox state was less oxidized in PHD1−/− neurons (Figure 4G).

Confirmation of the PHD1-Deficient Phenotype

Also in “aged” embryonic cortical neurons, cultured for 21 DIV as a surrogate model of adult neurons (Martin et al., 2008), PHD1 deficiency reduced glycolysis (by 25 ± 0.5%; n = 4; p < 0.05) while increasing oxPPP (by 2.2 ± 0.4-fold; n = 3; p < 0.05). In addition, using established methods (Ayala et al., 2006; Buescher et al., 2015), we infused [1,2-13C]glucose in adult conscious and unrestrained mice and measured in microdissected hippocampi the mass isotopomer distribution in pentose-5-phosphate sugars (pool of ribose-5-P and other pentoses) at the interface between the oxPPP and non-oxPPP (i.e., the non-oxidative branch of the PPP that generates ribose-5-P from glycolytic intermediates—however, without production of NADPH). We used the ratio of the M1 versus M2 labeling of pentose-5-phosphate as a parameter of the importance of the oxPPP versus non-oxPPP (Figure 5A). The M1 versus M2 labeling of pentose-5-phosphates was enriched more in PHD1−/− than WT hippocampi (0.97 ± 0.03 in PHD1−/− versus 0.87 ± 0.01 in WT hippocampi; n = 4–5; p < 0.05). Thus, similar to cultured neurons, the importance of the oxPPP was enhanced in the neuron-enriched hippocampus of PHD1−/− mice in vivo. Further, PHD1−/− mice showed lower GSSG levels in the ischemic hemisphere, indicating that PHD1 deficiency enables the ischemic brain to improve its redox maintenance (Figure 5B).

Ischemic Protection in PHD1−/− Neurons Is HIF Independent

We investigated if the effects of PHD1 deficiency relied on PHD1’s hydroxylase activity. We thus transduced PHD1−/− neurons with a vector expressing WT PHD1 (PHD1WT) or a hydroxylase-inactive PHD1 mutant (PHD1D311A) (McNeill et al., 2002) (Figure S5A). Re-expression of PHD1WT, but not PHD1D311A, in PHD1−/− neurons aggravated neuronal death compared to control PHD1−/− neurons after oxygen-nutrient deprivation (Figure 6A), indicating that inhibition of the hydroxylation activity of PHD1 mediates its neuroprotective effect.

Next, we explored the involvement of HIF-1α or HIF-2α. Immunoblotting showed that HIF-1α protein levels were not elevated in normoxic PHD1−/− neurons or PHD1−/− brain (Figures 6B and 6C), while HIF-2α protein was not detectable. Levels of HIF-1α transcriptional targets Vegta, Enolase, and Pdgf1 were not different either (Figure S5B). PHD1 deficiency did not also interfere with the hypoxic stabilization of HIF-1α, as evidenced by the similar levels of HIF-1α protein in PHD1−/− and WT brains (n = 3).
enhanced NF-κB activity in non-neuronal cell types, putatively via reduced hydroxylation and hence reduced degradation of IκB kinase 2 (IKK2) (Scholz and Taylor, 2013). IKK2 activates the NF-κB pathway by phosphorylation of IκBα, chaperone of the NF-κB transcription factor p65, resulting in degradation of IκBα and hence release of p65. Use of a NF-κB luciferase reporter showed that PHD1 deficiency increased the NF-κB promoter activity (Figure 6F), while protein levels of p65 were also elevated in PHD1−/− neurons (Figures 6G and S5C). Further, silencing of RelA (p65) reduced the oxPPP flux and TIGAR levels in PHD1−/− neurons (Figures 6H and 6I). These data indicate that NF-κB signaling downstream of PHD1 deficiency acts as metabolic regulator of the oxPPP flux via expression of TIGAR.

ICV Delivery of Anti-PHD1 Oligonucleotides Protects against Brain Ischemia
To test the translational potential of PHD1 inhibition in the brain, we used antisense oligonucleotides (ASOs). Incubation of cultured neurons with the anti-PHD1 ASO lowered Phd1 mRNA levels by 84% without affecting transcript or protein levels of PHD2 or PHD3 (Figures 7A and 7B). Intracerebroventricular (ICV) delivery of anti-PHD1 ASO lowered brain Phd1 mRNA levels (Figure 7C), reduced infarct size (Figures 7D–7G), and improved the performance in the adhesive tape removal test (Figures 7H and 7I).

DISCUSSION
The main findings of our study are that PHD1 is an important regulator of neuronal metabolism, providing protection against ischemic redox imbalance. The lack of a clear vascular phenotype in PHD1−/− brains is compatible with previous reports in the brain and other organs in PHD1−/− mice, in which vascular effects were not observed either (Aragónés et al., 2008; Chen et al., 2012; Schneider et al., 2009).

PHD1 Orchestrates Neuronal Glucose Metabolism
Neurons rely on oxidative glucose metabolism, but the metabolic adaptations during stroke have remained largely enigmatic. We show that PHD1 is a key regulator of glucose metabolism in neurons and orchestrates a protective response against an ischemic insult. Unexpectedly, at least based on previous results that PHD1 deficiency acts as metabolic regulator of the oxPPP flux via expression of TIGAR.

Possible Molecular Mechanism of PHD1 Deficiency
We then sought to provide further insight into the underlying molecular mechanism. Reduced PHD1 activity has been linked to by a similar increase in HIF-1α protein levels and transcriptional targets between the two genotypes in hypoxia (Figures 6B and S5B). Notably, silencing of HIF-1α or HIF-2α did not affect TIGAR transcript levels in PHD1−/− neurons (mRNA copies per 10^3 copies HPRT: 12.2 ± 3.0 in Ctrl PHD1−/−; 13.1 ± 2.8 in HIF-1αKD PHD1−/− and 12.7 ± 3.4 in HIF-2αKD PHD1−/− neurons, n = 5, p = NS). Also, silencing of HIF-1α, HIF-2α, or HIF-1β (which results in blockade of both HIF-1α and HIF-2α signaling) did not abolish the protection against oxygen-nutrient deprivation by PHD1 deficiency (Figures 6D and 6E). All these data suggest a HIF-independent mechanism of the PHD1-mediated protection.

Figure 3. PHD1 Deficiency Alters Neuronal Metabolism
(A) Glycolytic flux in WT and PHD1−/− neurons (n = 8).
(B) Glucose consumption in WT and PHD1−/− neurons (n = 3).
(C) Glucose oxidation in WT and PHD1−/− neurons (n = 7).
(D) Mitochondrial oxygen consumption rate, baseline (OCRBas) in WT and PHD1−/− neurons (n = 5).
(E) Relative change of OCRMAX (OCR sensitive to uncoupler dinitrophenol) and OCRATP (OCR sensitive to oligomycin) compared to OCRBas (dotted line) in WT and PHD1−/− neurons (n = 3).
(F) Glutamine oxidation in WT and PHD1−/− neurons (n = 8). All quantitative data are mean ± SEM, *p < 0.05. See also Figure S3 and Table S2.
hepatocytes relied on HIF-activation (Aragonez et al., 2008; Schneider et al., 2009), while this was not the case in PHD1−/− neurons. Hence, PHD1 has a distinct role in neurons as compared to other cell types, and neurons reprogram their metabolism in a (perhaps even unique) PHD1-dependent manner.

Neurons lacking PHD1 shunted more glucose into the oxPPP. This pathway is critical for redox homeostasis by generating NADPH, necessary to replenish GSH (Stanton, 2012). In agreement, PHD1−/− neurons had an improved capacity to scavenge oxygen radicals. Given that oxidative...
stress in brain ischemia can contribute to neuronal death (Chan, 2001; Moskowitz et al., 2010), the increase in oxPPP flux can confer protection. Importantly, PHD1 deficiency promoted the oxPPP flux in not only normoxic, but also in hypoxic conditions, and it better preserved the redox state of glutathione in ischemic stress. In fact, unlike WT neurons, PHD1−/− neurons still had a residual oxPPP flux during hypoxia. Even though smaller than in normoxia, this residual flux was of vital importance to secure sufficient redox homeostasis during hypoxic stress. These findings identify PHD1 as a regulator of the oxPPP and the associated oxygen radical scavenging capacity.

The role of the PPP in stroke has received little attention. G6PD is the rate-limiting enzyme of the oxPPP, which is upregulated in ischemia (Li et al., 2014), while TIGAR promotes the oxPPP at the expense of glycolysis. In line with our findings, inhibition of the oxPPP flux by silencing G6PD aggravated stroke outcome (Zhao et al., 2012), while TIGAR protects against ischemic brain injury, though oxPPP flux measurements were not performed in these studies (Cao et al., 2015; Li et al., 2014). Some evidence suggests a contextual role of the PPP, attributing opposing fates to NADPH. As a reducing co-factor of glutathione reductase, NADPH improves the anti-oxidant capacity, but as an electron donor of NADPH oxidase, NADPH can also generate superoxides (Stanton, 2012), explaining the divergent effects of PPP manipulation on ischemic neuroprotection (Cao et al., 2015; Herrero-Mendez et al., 2009; Li et al., 2014; Suh et al., 2008; Zhao et al., 2012). Regardless of these additional possible effects, regulation of the oxPPP by PHD1 deficiency resulted in improved—not impaired—anti-oxidant capacity.

**Mechanisms of oxPPP Regulation by PHD1**

Our study implies a role of NF-κB signaling downstream of PHD1 in the regulation of the oxPPP flux in neurons. In line with our findings, NF-κB signaling has been reported to lower glycolysis (Mauro et al., 2011). Our findings also indicate that the neuroprotective effect of PHD1 deficiency relies on the lack of hydroxylation activity of PHD1, but not on HIF-signaling, likely because HIF-1α levels are more importantly controlled by PHD2 than by PHD1 (Appelhoff et al., 2004; Myllyharju, 2013). Another reason why HIF-1α levels were not upregulated in PHD1−/− neurons may relate to the finding that ROS levels were decreased in PHD1−/− neurons. Since ROS inactivate PHDs (Quaegebeur and Carmeliet, 2010), reduced ROS levels are expected to result in less inactivated PHD2 and PHD3, which would favor the degradation of HIF-1α. Hence, this mechanism is expected to counteract any possible upregulation of HIF-1α by loss of PHD1.

**Ischemia Tolerance in PHD1−/− Brain: Distinct from Hypometabolism?**

We also considered if basal hypometabolism might explain the ischemia tolerance in PHD1−/− neurons, as various lines of evidence suggest such a link. First, hibernating animals cope with oxygen starvation by depressing oxidative metabolism (often by lowering oxygen consumption by >50-fold) and by reducing protein synthesis and other energy-consuming processes (Fraisl et al., 2009; Larson et al., 2014). Second, in the mammalian brain, hypoxia signaling has been implicated in inducing hypometabolism and other putatively protective responses, elicited by ischemic preconditioning (Quaegebeur and Carmeliet, 2010), even though a direct contribution to neuroprotection in acute ischemic events remains outstanding.

However, the ischemia tolerance of PHD1−/− neurons is distinct from the aforementioned phenomena. Indeed, loss of PHD1 in neurons is not accompanied by a reduction in oxygen consumption or energy conservation (as evidenced by the maintained Na+/K+ pump activity, dendritic branching, protein and RNA synthesis). Although we cannot exclude a slight energy hypometabolism in PHD1−/− neurons (below the detection threshold of our techniques), we were not able to pick up genotypic differences when measuring energy consumption and production. Instead, PHD1 deficiency in neurons is characterized by reduced glycolysis and glucose oxidation, but increased oxPPP and glutamine oxidation, and maintained lactate oxidation and oxygen consumption (i.e., metabolic changes that are opposite to those accompanying hypometabolism during hibernation or ischemic preconditioning). Such a metabolic reprogramming underlying ischemia tolerance has not been reported previously.
Translational Potential of PHD1 Inhibition

Our data show that hypoxia increases glycolysis, which can deprive neurons from a vitally important oxPPP flux. Interestingly, in cell culture and animal models of neurodegenerative disorders, signs of increased glycolysis have been sporadically described (D’Alessandro et al., 2011; Ferreira et al., 2011; Rodriguez-Rodriguez et al., 2012; Yao et al., 2009), raising the speculative question whether a similar metabolic shift is taking place in these conditions and whether PHD1 inhibition would be beneficial as well in these diseases.

Translational Potential of PHD1 Inhibition

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Silencing of PHD1 by ICV delivery of ASOs prior to stroke protected against brain ischemia, thus phenocopying the
genetic data. However, a clinically more relevant strategy would be to administer a PHD1 inhibitor after stroke onset. Unfortunately, we had no access to a PHD1-selective chemical inhibitor to test how rapidly PHD1 blockade would induce ischemia tolerance. Despite this limitation, our prevention studies raise the question if PHD1 inhibition might be clinically useful to diminish neuronal damage in conditions where the risk of cerebral ischemia is considered high, such as upon a critical carotid stenosis or following a subarachnoid hemorrhage, though possible clinical benefit of such strategy awaits further study.

A therapy based on blocking PHD1 may also differ from previously tested stroke targets relying on blocking oxygen radicals by administering anti-oxidants. Indeed, many of those treatment strategies neglected the importance of physiological signaling by oxygen radicals in neurons, partly explaining the poor translational success (Moskowitz et al., 2010). PHD1 inhibition is a fundamentally different and perhaps more appealing approach, as it relies on switching on an endogenous protective mechanism. Future research will clarify if PHD1 inhibition is valid for neuroprotection when initiated after stroke onset.

EXPERIMENTAL PROCEDURES

Detailed methods are described in the Supplemental Information.

Mouse Strains

PHD1−/− (Aragonés et al., 2008), PHD2lox/lox (Mazzone et al., 2009), and PHD3−/− (Aragonés et al., 2008) mice were described. PHD3lox/lox mice were intercrossed with Nestin-Cre mice (Tronche et al., 1999) to obtain neuronal-specific PHD2-deficient mice. Housing and experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the University of Leuven, Belgium.

Stroke Model

Brain ischemia was induced using the pMCAO model (Kuraoka et al., 2009). Infarct size was analyzed at 24 hr after ischemia by vital dye staining of 1 mm coronal brain slices (Bederson et al., 1986), and infarction area was calculated using ImageJ. Other experiments were performed at the Department of Neurology, Ghent University Hospital, Ghent, Belgium.
calculated as the ratio of the unstained area over the total bihemispheric area, using ImageJ. Functional impact of the stroke was tested using the adhesive tape removal test (Bouet et al., 2009).

Brain Vasculature
Arteries were analyzed by immunostaining for α-SMA; vessel perfusion after tail vein injection with fluorescein isothiocyanate (FITC)-conjugated dextran; pial collateral circulation via vascular corrosion casting (Krucker et al., 2006) and counting of the number of collaterals per hemisphere. The stroke-induced perfusion deficit in the brain was measured by MRI at 2 and 24 hr after pMCAO.

Neuronal Culture
Cortical neurons were prepared from cortices of embryonic day 14.5 or 15.5 mice, pooled from one litter (Thathiah et al., 2009). Gene silencing was done after 2 days using gene-specific lentiviral shRNA vectors. Oxygen-nutrient deprivation was initiated after 7 days in culture by exposure for 2 hr to 0.1% oxygen in nutrient-deprived medium followed by re-exposure to ambient air and regular medium.

Metabolic Flux Assays
Glycolysis, oxPPP, glucose, glutamine, and lactate oxidation were measured using radiolabeled tracers. Glucose consumption was defined as the difference in glucose concentration in the medium after 24 hr and was measured via liquid chromatography-mass spectrometry (LC-MS). OCR was measured using the extracellular flux analyzer XF24 (Seahorse Bioscience). ROS scavenging was measured using CM-H2DCF. GSSG/GSH levels were determined using LC-MS. ATP/ADP/AMP levels were determined using HPLC. Intracellular Na⁺ concentration was monitored using SBFi-based microfluorimetry.

RNA, DNA, and Protein Analysis
Gene expression was analyzed by qRT-PCR, using premade primer sets (Applied Biosystems). The amount of mtDNA relative to nuclear genomic DNA was determined by qPCR using primers for cytochrome b (mitochondrial) and RPL13A (nuclear). Immunoblotting was done using the antibodies listed in the Supplemental Information with signal detection using the ECL system (Amer sham Biosciences, GE Healthcare). Immunocytochemistry was done on cortical neurons grown for 7 days on Wilco-Dish glass bottom dishes (Ibidi), using the antibodies listed in the Supplemental Information.

ICV Delivery of Oligonucleotides
ASOs against murine PHD1 mRNA were designed by Isis Pharmaceuticals. Mice were treated with ASOs by ICV infusion for 10 days using Alzet osmotic pumps (Cupertino) connected with a catheter to a brain infusion cannula. 2 weeks after the infusion, pMCAO was performed.

Statistics
All data are mean ± SEM of the indicated number of experiments or mice, unless otherwise noted (median). For the in vitro data, the “n” values indicate the number of independent experiments conducted with different cultures of WT and PHD1−/− cortical neurons (independent isolations). For the in vivo experiments, two-sided Student’s t tests were used and done using Prism v5.0, considering p < 0.05 statistically significant. In some PHD1−/− mice, no infarct was visible after ligation of the middle cerebral artery (indicating complete protection against ischemia). In these cases, we used non-parametrical Mann-Whitney-U test to determine statistical differences. For in vitro experiments, statistical significance between groups was calculated on n pooled independent experiments using linear mixed-effect model statistics to correct for effects by inter-experimental variation (Schoors et al., 2015). Calculations were done using R and the lme4 package. In all cases, the condition was entered as fixed effect. p values were obtained with the Kenward-Roger F-test for small mixed effect model datasets using lmerTest package. Again, p < 0.05 was considered statistically significant. For the time course experiments with CM-H2DCF fluorescence, we used linear mixed-effect model statistics to determine the slope and statistical differences between slopes.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.12.007.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST
G.H. and C.F.B. are employees of Isis Pharmaceuticals Inc. and could benefit if a therapeutic product for treatment of ischemic stroke results from this work.

ACKNOWLEDGMENTS
The authors thank Matthew Vander Heiden for providing training in in vivo 13C-glucose infusions and Carlos Dotti for advice on the use of aged cultured neurons. Funding support: Research Foundation–Flanders (FWO) (A.Q., I.S., B.G., K.D.B., and S.-M.F.); FP7 Marie Curie Intra-European Fellowship (I.D. and F.B.); Pegasus Marie Curie–FWO (R.Q.); senior clinical investigator of FWO Flanders (R.L.); Agency for Innovation by Science and Technology in Flanders (FWO) (B.C. and S.S.). The work of P.C. is supported by a Belgian Science Policy grant (IUAP P7/03), long-term structural Methusalem funding by the Flemish Government, grants from the FWO (GO671.12N, 1.5.244.11N) and Foundation Leduq Transatlantic Network (ARTEMIS), funding by “A cure for ALS” from ALS Liga Belgium, Motor Neuron Disease Association, ALS Association (ID#C44128), the European Research Council (ERC grant agreement n° 340429) and the European Union’s Seventh Framework Programme (FP7/ 2007-2013; grant agreement n° 259867), and the Leuven University Fund - Opening the Future. W.R. is supported through the E. von Behring Chair for Neuromuscular and Neurodegenerative Disorders & Geneekundige Stichting Koningin Elisabeth (G.S.K.E.).

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REFERENCES


Supplemental Information

Deletion or Inhibition of the Oxygen Sensor PHD1

Protects against Ischemic Stroke

via Reprogramming of Neuronal Metabolism

Annelies Quaegebeur, Inmaculada Segura, Roberta Schmieder, Dries Verdegem, Ilaria Decimo, Francesco Bifari, Tom Dresselaers, Guy Eelen, Debapriya Ghosh, Shawn M. Davidson, Sandra Schoors, Dorien Broekaert, Bert Cruys, Kristof Govaerts, Carla De Legher, Ann Bouché, Luc Schoonjans, Matt S. Ramer, Gene Hung, Goele Bossaert, Don W. Cleveland, Uwe Himmelreich, Thomas Voets, Robin Lemmens, C. Frank Bennett, Wim Robberecht, Katrien De Bock, Mieke Dewerchin, Bart Ghesquière, Sarah-Maria Fendt, and Peter Carmeliet
**SUPPLEMENTAL INFORMATION**

**SUPPLEMENTAL DATA**

**FIGURE S1: RELATED TO FIGURE 1**

**A,B,** TTC stained brain slices showing unstained infarct zone (white) from WT (A) and PHD1<sup>−/−</sup> mice (B) on a 129S6 background, 24 hours after pMCAO. **C,** Quantification of the stroke area (% of total bihemispheric area) (n = 9 for WT, n = 6 for PHD1<sup>−/−</sup>, * p < 0.05 by Mann-Whitney U test; difference in medians: -1.34 vs WT, CI 95%: -2.64 to -0.43). Quantitative data are mean ± SEM.
**Figure S2:** Related to Figure 2

**A,B,** Representative images of α-SMA immunostained brain sections, visualizing arterioles in WT (A) and PHD1−/− brains (B). **C,** Quantification of α-SMA positive area as percentage of total brain area (n = 4 for WT, n = 5 for PHD1−/−, p = NS; difference in means: +0.016 vs WT, CI 95%: -0.067 to 0.098). **D,** mRNA expression levels of Phd2 and Phd3 in isolated WT and PHD1−/− neurons (n = 3, p = NS). **E,** Representative immunoblots of PHD2 and PHD3 protein levels in WT and PHD1−/− neurons. Densitometric quantification of independent experiments (% of β-actin loading control): 71 ± 36% in WT and 63 ± 36 in PHD1−/− neurons for PHD2, 39 ± 23% in WT and 38 ± 23 in PHD1−/− neurons for PHD3, n = 4, p = NS). **F,G,** Representative Nissl stained coronal brain sections of E14.5 WT (F) and PHD1−/− (G) embryos. The PHD1−/− embryonic brain is comparable to the WT embryonic brain in appearance, cortical thickness (arrow) and lateral extension of the cortex (dashed line), indicative of a normal brain cytoarchitecture. **H,I,** Quantifications of the cortical thickness (H) and lateral extension of the cortex (I) in E14.5 WT and PHD1−/− embryos (n = 3, p = NS; difference in means: +15.6 vs WT, 95% CI: -14.9 to 46.1 for cortical thickness (H); and difference in means: +5.23 vs WT, 95% CI: -110 to 121 for lateral extension (I)). **J,K,** Representative MAP2 immunostaining of WT (J) and PHD1−/− (K) neuronal cultures showing normal morphological appearance. All quantitative data are mean ± SEM.
**Table S1: Gene expression analysis of neurotrophic factors (related to Figure 2)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT Neurons</th>
<th>PHD1&lt;sup&gt;−/−&lt;/sup&gt; Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bdnf</em></td>
<td>0.0179 ± 0.0109</td>
<td>0.0186 ± 0.0097</td>
</tr>
<tr>
<td><em>Ngf</em></td>
<td>0.0222 ± 0.0064</td>
<td>0.0228 ± 0.0033</td>
</tr>
<tr>
<td><em>Nt3</em></td>
<td>0.1306 ± 0.0145</td>
<td>0.1096 ± 0.0084</td>
</tr>
<tr>
<td><em>p75</em></td>
<td>0.0994 ± 0.0185</td>
<td>0.0966 ± 0.0229</td>
</tr>
<tr>
<td><em>TrkB</em></td>
<td>2.5642 ± 0.300</td>
<td>2.5292 ± 0.3001</td>
</tr>
<tr>
<td><em>TrkC</em></td>
<td>1.6391 ± 0.1821</td>
<td>1.6912 ± 0.2192</td>
</tr>
<tr>
<td><em>Vegf-a</em></td>
<td>1.0260 ± 0.4785</td>
<td>1.1091 ± 0.3366</td>
</tr>
<tr>
<td><em>Vegf-b</em></td>
<td>1.6121 ± 0.3207</td>
<td>1.6121 ± 0.4357</td>
</tr>
<tr>
<td><em>Vegfr2</em></td>
<td>0.4526 ± 0.4017</td>
<td>0.2252 ± 0.1330</td>
</tr>
</tbody>
</table>

Quantitative RT-PCR analysis of the mRNA levels of different neurotrophic factors and their receptors in cultured WT and PHD1<sup>−/−</sup> neurons. Values represent number of mRNA copies/10<sup>3</sup> copies of β-actin (mean ± SEM, n = 3-6, p = NS). *Bdnf*: Brain-derived neurotrophic factor; *Ngf*: Nerve growth factor; *Nt3*: Neurotrophin 3; *p75*: p75 neurotrophin receptor; *TrkB* and *TrkC*: tyrosine receptor kinase B and C; *Vegf-a* and *Vegf-b*: vascular endothelial growth factor A and B; *Vegfr2*: Vascular endothelial growth factor receptor 2.
**Figure S3: Related to Figure 3**

A-D, Representative images of neuronal cultures, double-immunostained for TOMM20 (red; A-D) and MAP2 (green; B,D), visualizing the mitochondria and dendritic arbors, respectively, in WT (A,B) and PHD1−/− neuronal cultures (C,D); panels B,D represent the merged image. E, Quantification of mitochondrial area (i.e. TOMM20 positive area) per cell (i.e. MAP2 positive area) (n = 3, p = NS). F, The Seahorse extracellular flux analyzer allows the measurement of different bioenergetics parameters by sequential injection of different inhibitors of the mitochondrial electron transport chain. Antimycin-A injection allows the calculation of non-mitochondrial OCR and the baseline mitochondrial respiration (OCR\text{BAS}). Oligomycin injection allows measuring of respiration coupled to ATP synthesis (OCR\text{ATP}) and maximal respiration (OCR\text{MAX}) is induced by the uncoupler 2,4-dinitrophenol (DNP). G, Levels of AMP, ADP and ATP in WT and PHD1−/− neurons (a.u.: arbitrary units, n = 3; p = NS). H,I, Representative images of tandem tomato (tdT)-transfected WT (H) and PHD1−/− (I) neurons after 7 days in culture (shown in gray scale). J, Sholl analysis quantifying neuronal arborization (a process relying on mitochondrial ATP production), expressed as the number of intersections with an overlay of concentric circles positioned at the indicated distances from the soma (n = 52 neurons per genotype; p = NS). K-O, Similar activity of the ATP-consuming Na\textsuperscript{+}/K\textsuperscript{+} pump (a main consumer of energy in neurons) in WT and PHD1−/− neurons, indicating that PHD1 deficiency did not induce energy hypometabolism. Intracellular sodium levels in WT and PHD1−/− neurons in baseline (K), and time curves of intracellular sodium concentration ([Na\textsuperscript{+}]) as measured by SBFI-fluorescence in WT (L) and PHD1−/− (M) neurons, showing the reversible rise in [Na\textsuperscript{+}] when exposing the cells for 10 minutes to a K\textsuperscript{+}-free buffer (horizontal bar) instead of the standard K\textsuperscript{+}-containing buffer; quantifications show the amplitude of the peak of the increase in [Na\textsuperscript{+}] (N) and the time constant of the decay (τ\text{decay}) obtained by fitting a monoexponential function to the [Na\textsuperscript{+}] decay phase following readdition of extracellular K\textsuperscript{+} (O) (n = 3; p = NS). P-U, Panels P-U show experimental evidence of maintained RNA and protein synthesis, indicating that PHD1 deficiency did not induce energy hypometabolism. P-R, Representative images of ethinyl uridine (EU, red) staining reflecting similar RNA synthesis in WT (P) and PHD1−/− (Q) neurons, quantified in panel R as EU\textsuperscript{+} intensity per
nucleus (DAPI stained, blue) (n = 3; p = NS). Merged images with MAP2 immunostaining (green) and DAPI staining are shown. **S-U**, Representative images of L-homopropargylglycine (HPG, red) staining showing similar protein synthesis in WT (S) and PHD1−/− (T) neurons (MAP2+ staining, green), quantified in (U) as HPG+ intensity per MAP2+ area (n = 2; p = NS). A.u., arbitrary units.
**TABLE 2: GENE EXPRESSION ANALYSIS OF GLYCOLYTIC ENZYMES (RELATED TO FIGURE 3)**

<table>
<thead>
<tr>
<th>GENE</th>
<th>WT NEURONS</th>
<th>PHD1&lt;sup&gt;−/−&lt;/sup&gt; NEURONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glut-1</td>
<td>11.03 ± 4.53</td>
<td>8.52 ± 3.36</td>
</tr>
<tr>
<td>Glut-3</td>
<td>35.96 ± 6.20</td>
<td>35.54 ± 6.22</td>
</tr>
<tr>
<td>Hk1</td>
<td>178.50 ± 12.32</td>
<td>206.22± 16.89</td>
</tr>
<tr>
<td>Pfk1</td>
<td>65.36 ± 4.29</td>
<td>63.45 ± 4.87</td>
</tr>
<tr>
<td>Pfkfb3</td>
<td>15.79 ± 14.42</td>
<td>14.44 ± 13.51</td>
</tr>
<tr>
<td>Gapdh</td>
<td>69.09 ± 45.13</td>
<td>69.86 ± 47.13</td>
</tr>
<tr>
<td>Pgam2</td>
<td>19.29 ± 4.56</td>
<td>22.10 ± 5.91</td>
</tr>
<tr>
<td>Eno</td>
<td>49.05 ± 12.27</td>
<td>49.05 ± 12.27</td>
</tr>
<tr>
<td>Ldh-a</td>
<td>441.20 ± 53.94</td>
<td>509.05 ± 28.29</td>
</tr>
<tr>
<td>Pdk1</td>
<td>79.44 ± 46.60</td>
<td>87.92 ± 57.45</td>
</tr>
</tbody>
</table>

Quantitative RT-PCR data of the mRNA levels of different glycolytic genes in WT and PHD1<sup>−/−</sup> neurons in baseline conditions. Values represent number of mRNA copies/10<sup>3</sup> copies of HPRT (mean ± SEM, n = 3-4, p = NS). *Glut*: glucose transporter; *Hk1*: hexokinase; *Pfk*: phosphofructokinase; *Pfkfb*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; *Gapdh*: glyceraldehyde-3-phosphate dehydrogenase; *Eno*: Enolase; *Pgam2*: phosphoglycerate mutase 2; *Ldh-a*: lactate dehydrogenase a; *Pdk1*: pyruvate dehydrogenase kinase, isoform 1.
**Figure S4: Related to Figure 4**

A, Levels of glycolytic intermediates in WT and PHD1−/− neurons (n = 3, p = NS). G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-biphosphate; PEP, phosphoenolpyruvate. B, G6PD mRNA expression levels (expressed as mRNA copies/10^3 copies β-actin) in WT and PHD1−/− neurons (n = 3, p = NS). C, oxPPP flux in WT neurons transduced with a lentiviral vector, expressing an empty control (ctrl) or human TIGAR (hTIGAR) (n = 4). D, oxPPP flux in PHD1−/− neurons transduced with lentiviral vectors expressing a scrambled control (ctrl) or shTIGAR (TIGAR^KD) (n = 5). E-G, Representative images of mitoSOX stained WT (E) and PHD1−/− neurons (F), revealing mitochondrial superoxide radical levels. Neurons were counterstained with DAPI to visualize cell nuclei. Panel G shows the quantification of mitoSOX fluorescence per cell (n = 3; p = NS; 100-200 cells per isolation were quantified). H, Schematic representation of the effect of silencing G6PD (shG6PD) on the PPP flux, the conversion of oxidized to reduced glutathione, and the detoxification of H_2O_2. I, oxPPP flux in PHD1−/− neurons transduced with lentiviral vectors, expressing scrambled control (ctrl) or shG6PD (G6PD^KD) (n = 3). J,K, Glucose (J) and glutamine oxidation (K), measured in hypoxic WT and PHD1−/− neurons (expressed relative to the values obtained in normoxic WT neurons, n = 4). All quantitative data are mean ± SEM. * p < 0.05.
**Table S3:** Gene expression analysis of anti-oxidant enzymes (related to Figure 4)

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT Neurons</th>
<th>PHD1&lt;sup&gt;−/−&lt;/sup&gt; Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gclc</td>
<td>20.94 ± 1.94</td>
<td>25.63 ± 8.76</td>
</tr>
<tr>
<td>Gpx1</td>
<td>383.68 ± 77.43</td>
<td>398.65 ± 39.53</td>
</tr>
<tr>
<td>Gpx4</td>
<td>201.26 ± 16.16</td>
<td>231.60 ± 18.66</td>
</tr>
<tr>
<td>Prdx1</td>
<td>258.96 ± 43.85</td>
<td>241.52 ± 35.67</td>
</tr>
<tr>
<td>Prdx3</td>
<td>142.73 ± 36.97</td>
<td>131.77 ± 39.92</td>
</tr>
<tr>
<td>Prdx4</td>
<td>158.40 ± 57.18</td>
<td>154.41 ± 20.80</td>
</tr>
<tr>
<td>Sesn1</td>
<td>11.28 ± 1.11</td>
<td>12.07 ± 0.72</td>
</tr>
<tr>
<td>Sesn2</td>
<td>10.08 ± 2.23</td>
<td>10.47 ± 1.22</td>
</tr>
<tr>
<td>SOD1</td>
<td>655.87 ± 66.86</td>
<td>642.80 ± 93.91</td>
</tr>
<tr>
<td>SOD2</td>
<td>212.56 ± 68.75</td>
<td>176.86 ± 48.09</td>
</tr>
</tbody>
</table>

Quantitative RT-PCR data of the mRNA levels of different anti-oxidant genes in WT and PHD1<sup>−/−</sup> neurons in baseline conditions. Values represent number of mRNA copies/10<sup>3</sup> copies of HPRT (mean ± SEM, n = 3-6, p = NS). Gclc: glutamate-cystein ligase catalytic subunit; Gpx: glutathione peroxidase; Prdx: peroxiredoxin; Sesn: sestrin; SOD: superoxide dismutase.
**Figure S5: Related to Figure 6**

A, PHD1 mRNA expression levels in control WT and PHD1\(^/-\) neurons with or without transduction with lentiviral vectors expressing WT PHD1 (PHD1\(^{WT}\)) or an hydroxylation-defective mutant PHD1 (PHD1\(^{D311A}\)) (n = 3). B, mRNA expression levels of HIF1\(\alpha\) transcriptional targets in normoxic and hypoxic WT or PHD1\(^/-\) neurons (n = 3-5). Eno1, enolase; Pdk1, pyruvate dehydrogenase kinase 1; Vegfa, vascular endothelial growth factor A. C, Representative immunoblots of total p65 levels in WT and PHD1\(^/-\) neurons. Densitometric quantification of independent experiments (% of control \(\beta\)-actin): 76.6 ± 8.4% in WT and 95.9 ± 8.4% in PHD1\(^/-\) neurons, n = 3, p < 0.05. All quantitative data are mean ± SEM.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

MOUSE STRAINS USED IN THIS STUDY

PHD1\(^{-/-}\) (Aragones et al., 2008), PHD2\(^{lox/lox}\) (Mazzone et al., 2009) and PHD3\(^{-/-}\) (Aragones et al., 2008) mice were previously reported. Given the embryonic lethality of homozygous global PHD2 deficiency (Takeda et al., 2006), neural specific PHD2 deficient mice were used, generated by intercrossing Nestin-Cre mice (Tronche et al., 1999) with PHD2\(^{lox/lox}\) mice (Mazzone et al., 2009). The Nestin-Cre mice were on a C57Bl/6N background and were a kind gift from Dr. R. Klein (MPI, Germany). Nestin-Cre mice were intercrossed with PHD2\(^{lox/lox}\) mice. Nestin-Cre\(^{+}\) PHD2\(^{lox/lox}\) (PHD2\(^{NKO}\)) mice were compared with littermates Nestin-Cre\(^{-}\) PHD2\(^{lox/lox}\) (ctrl). WT C57Bl/6N and 129S mice were obtained from the KU Leuven animal facility. For the in vivo experiments, only male mice, aged 8-11 weeks, littermates or age-matched mice on a pure C57Bl/6N or 129S background were used. Housing and experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the University of Leuven, Belgium.

ISCHEMIC STROKE VIA PERMANENT MIDDLE CEREBRAL ARTERY OCCLUSION

Brain ischemia was induced in 8-11 weeks old male wild type and PHD1\(^{-/-}\) mice using the permanent middle cerebral artery occlusion (pMCAO) model (Kuraoka et al., 2009). Both C57Bl/6N and 129S6 backgrounds were used, as indicated. Under the surgical microscope, a skin incision was made between the left orbit and tragus. The zygomatic arch was removed and temporal muscle retracted laterally. A 2-mm burr hole was made with a microdrill through the outer surface of the semitranslucent skull over the visually identified middle cerebral artery. The dura was carefully opened and the M1 branch of the MCA was exposed and ligated producing a permanent ligation of the distal middle cerebral artery. The wound was sutured and temperature was controlled by keeping the mice on a heating plate at 37 °C until they regained full consciousness. Mice were randomly assigned to treatments and the procedure was performed by an experimenter blinded for the genotypes.
**HISTOLOGICAL ANALYSIS OF INFARCT SIZE**

At 24 hours after ischemia, mice were euthanized and brains were removed, sliced coronally at 1 mm intervals and stained by immersion in a 2% solution of the vital dye 2,3,5-triphenyltetrazolium hydrochloride (TTC) for 30 minutes at 37 °C. TTC will be reduced by active mitochondrial dehydrogenases, indicative of viable tissue, resulting in the formation of a deep-red product (formazan) (Bederson et al., 1986). For each coronal slice the unstained portion was delineated, and infarction area was calculated as the ratio of the unstained area (white) over the total bihemispheric area with Image J. The analysis was performed by an experimenter blinded for the genotypes and treatments.

**ADHESIVE TAPE REMOVAL TEST**

The performance in the adhesive tape removal test was used as a functional parameter to monitor the functional impact of the stroke as previously described (Bouet et al., 2009). Mice were trained during 7 days prior to stroke induction. A 3 mm x 4 mm piece of tape was attached to both forepaws. Time to sensation and to removal was recorded for both paws. Three separate measurements were done 1 day before stroke and at 1, 4, 8 and 11 days post-stroke. The analysis was performed by an experimenter blinded for the genotypes and treatment.

**MORPHOLOGICAL AND HISTOLOGICAL ANALYSIS OF BRAIN VASCULATURE**

To compare large arteries and arterioles in WT and PHD1⁻/⁻ mice, animals were perfused transcardially with saline followed by 4% PFA. Brains were dissected and post-fixed in 4% PFA overnight at 4°C, cryoprotected in sucrose 20% and embedded in optimal cutting temperature (OCT) embedding medium. Serial coronal cryosections of 10 μm thickness were incubated overnight with the primary antibody anti-αSMA-Cy3 (C6198, Sigma, 1/500). Slices were mounted with Prolong Gold + DAPI (Invitrogen). To assess vessel perfusion, WT and PHD1⁻/⁻ mice were injected via the tail vein with fluorescein isothiocyanate (FITC)-conjugated dextran (molecular weight 2x10⁶ Da, Sigma) shortly
before transcardial perfusion, and were thereafter processed for cryosectioning. Vessel perfusion after stroke was assessed by i.v. injection of fluorescein isothiocyanate (FITC)-conjugated dextran (molecular weight 2x10^6 Da, Sigma) of the mice 2 hours after ligating the middle cerebral artery. Shortly thereafter the mice were euthanized and transcardially perfused. Coronal cryosections through the non-stroked brain or through the stroke area and corresponding contralateral cortex area were stained with Alexa568-conjugated isolectin B4 (Vector Laboratories), visualizing the vessel wall. The ratio of dextran-positive area over the isolectin-positive area reflected the perfused vessels. Visualization of the \textbf{PIAL COLLATERAL CIRCULATION} in WT and PHD1\textsuperscript{+/-} mice was done via vascular corrosion casting as previously described (Krucker et al., 2006). The thoracic aorta was cannulated retrogradely and 1 ml of freshly prepared polyurethane resin (PU4ii, VasQtec) was infused into the cerebral vasculature. The PU4ii mixture was freshly prepared by adding 0.8 g of hardener (VasQtec) to PU4ii diluted in dimethylethylketone, immediately before the injection. Following resin injection, mice were anesthetized and perfused with 4% PFA. After solidification of the resin for 5 days at room temperature, brains were dissected and were repeatedly rinsed in 30% KOH. Images of the corrosion casting were captured by a Zeiss LSM 780 confocal microscope (Carl Zeiss). The number of collaterals was counted manually per hemisphere. Collaterals were defined as vessels connecting the tree of the anterior cerebral artery with the tree of the middle cerebral artery, or connecting one arteriole from the middle cerebral artery with an arteriole from the same vascular tree. All analyses were performed by an experimenter blinded for the genotypes.

\textbf{MAGNETIC RESONANCE IMAGING OF PERFUSION POST-STROKE}

Focal brain ischemia was induced in male 129S mice (8-10 weeks) as described before (Kuraoka et al., 2009). Mice were scanned at 2 hours and 24 hours post-surgery (anesthesia: isoflurane 1.2-1.4%, in medical air). MR images were recorded on a 9.4 T Biospec small animal MR system (20 cm horizontal bore, Bruker Biospin, Ettlingen, Germany) using a 7 cm linearly polarized resonator for transmission and an actively decoupled mouse brain surface coil for receiving (Rapid Biomedical, Rimpar, Germany).
T2w images (effective TE 50 ms; matrix 256x256; 300 µm slice thickness) were first acquired to assess the vasogenic lesion volume. Next perfusion (ASL) data were acquired from an axial slice location that covered the largest part of the T2w lesion using a FAIR approach (Kim, 1995; Kwong et al., 1995) and a RARE readout with the following specific parameters: TR 10 s, TE 5.3 ms, rare factor 72, FOV 2.5x2.5 cm, matrix 256x256, partial FT with 16 overscan, slice thickness 1 mm, inversion slab thickness 1.6 mm, 7 inversion times from 100-4000 ms, using a inversion hyperbolic secant of 14 ms (Paravison 5.1, Bruker).

Vasogenic lesion volumes were determined by manual delineation on the T2w images (Paravison 5.1). The FAIR perfusion data were first converted to quantitative perfusion images using the T1 difference method assuming an arterial T1 of 2.4s (Kober et al., 2008; Oosterlinck et al., 2011) implemented via a home-written Python (Python software foundation) scripts. Next area's with reduced perfusion were derived using an in-house developed Mevislab (Mevis Medical Solutions AG, Bremen, GE) processing pipeline using a threshold-based approach with the contra-lateral cortex as a reference region (threshold: cerebral blood flow, CBF ≤ 80%). All quantitative data are mean ±SEM.

**ISOLATION AND CULTURE OF MURINE CORTICAL NEURONS**

Cultures of cortical neurons were prepared as previously described (Thathiah et al., 2009). Briefly, neuron cultures from WT and PHD1−/− mice (both mixed Swiss/129S background) were isolated from the cortices of embryonic day 14.5 or 15.5 mice, pooled from one litter, plated on poly-D-lysine and laminin coated plates and maintained in serum-free Neurobasal media containing 25 mM glucose, supplemented with 0.5 mM glutamine and B27 supplement (all from Invitrogen). After 3 days in culture, 8 µM cytosine arabinoside (Sigma) was added to prevent non-neuronal proliferation. These cultures contained >98% MAP2⁺ neurons after 7 days in culture, as determined by staining for the glial marker GFAP, and the oligodendrocyte markers NG2 and Olig2. This fraction of contaminating non-neuronal cells (<2%) in the cultures did not differ between the two genotypes.
LENTIVIRAL TRANSDUCTIONS

Lentiviral shRNA vectors against glucose-6-phosphate dehydrogenase (G6PD), TIGAR, PHD2, PHD3, HIF-1α, HIF-2α, HIF-1β and Rel-A were ordered from the TRC 1.5 lentivirus-based shRNA library (Sigma). The shRNA ID numbers were TRCN0000041443 and TRCN0000041447 for G6PD, TRCN0000115292 and TRCN0000115294 for TIGAR, TRCN0000232222 for HIF-1α, TRCN0000082306 for HIF-2α, TRCN0000079929 and TRCN0000079931 for HIF-1β (combined), TRCN0000009740 for PHD2, TRCN0000009753 for PHD3, and TRCN0000055346 for Rel-A. A similar nonsense scrambled shRNA sequence was used as a negative control for all these experiments. Production of lentiviruses by transfection into 293T cells was as described (Carlotti et al., 2004). After 2 days in culture, the cultured neurons were exposed overnight to a multiplicity of infection (MOI) of 10 after which the medium was refreshed. Knockdown efficiency was assessed 4 days after transduction by qPCR.

To obtain overexpression of TIGAR at physiological levels, WT neurons were transduced with a lentiviral construct of human TIGAR at MOI 0.5 (Life Technologies, Mammalian Gene Collection ID4576725). Wild type PHD1 or hydroxylation-inactive mutant PHD1 were expressed in PHD1−/− neurons by transducing them with the corresponding lentiviral constructs at MOI 5 (McNeill et al., 2002). Neurons at 5 days in in vitro culture (DIV) were transfected with a tandem Tomato (tdT) expressing construct, using Lipofectamine2000 (Invitrogen), following manufacturer's instructions. To assess NF-kB promoter activity we co-transduced cells at DIV5 with a lentiviral NF-kB luciferase reporter (Cignal Lenti NFkB reporter, SABioscience, MOI 5), consisting of a tandem repeats of the NF-kB transcriptional response element and a renilla control reporter (Cignal™ Lenti TK-Renilla control, SABioscience, MOI 0.5) serving as an internal control. After 48 hours cells were lysed and luciferase and renilla enzymatic activity was measured in Microlumat LD96V Luminometer (Berthold Technologies) using the Dual Luciferase assay (Promega) as described by manufacture’s instructions. The ratio of firefly over renilla luminescence represents the NF-kB promoter activity in the cell lysates.
OXYGEN-NUTRIENT DEPRIVATION ASSAY IN VITRO

After 7 days in culture, neurons were exposed for 2 hours to 0.1% oxygen in nutrient-deprived medium (Dulbecco’s Modified Eagle’s Medium (D5030, Sigma) lacking glucose, glutamine and pyruvate, supplemented with 7.5 mM NaHCO₃ and 5 mM HEPES (pH=7). After this period of oxygen-nutrient deprivation, neurons were re-exposed to ambient air and regular nutrient-containing culture medium (Neurobasal medium supplemented with 0.5 mM glutamine and B27 supplement minus anti-oxidants, all from Invitrogen). The lack of anti-oxidants in the medium prevented masking a neuron-intrinsic improvement of ROS detoxification, which is crucial in all neuronal cell death assays relating to oxidative stress. Neuronal cell death was quantified 24 hours later by measuring LDH release in the culture medium using the Cytotoxicity Detection Kit (Roche Applied Science). LDH release after maximal lysis of control wells (with Triton-X) was used to calculate the relative cell death. At different time-points during reperfusion, cells were harvested for GSSG/GSH measurements (see below).

METABOLIC FLUX ASSAYS

Metabolic fluxes were measured using radiolabeled tracer glucose, glutamine and lactate (all tracers were from American Radiolabeled Chemicals). Postulating that metabolic changes might account for the protective effect, all metabolic measurements were performed after 7 days in culture (7 DIV), the time at which cultures are used for the oxygen-nutrient deprivation assay. GLYCOLYSIS was measured as ³H₂O formation using [5-³H]-D-glucose as previously described (De Bock et al., 2013). Briefly, at 7 DIV, cortical neurons were incubated for 2 hours with medium containing labeled [5-³H]-glucose (0.5 µCi/mmol). After this incubation period, the medium was transferred to glass vials, which were closed with rubber stoppers each containing a hanging well with a soaked (200 µl Milli-Q water) filter paper (1 cm x 6 cm) to capture the evaporated ³H₂O. The vials were incubated for 48 hours at 37°C. Afterwards, the filter papers were transferred to scintillation vials containing 5 ml of scintillation fluid (Lumasef 3087, Perkin Elmer) and counted with the Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer). GLUCOSE
OXIDATION was assessed as $^{14}$CO$_2$ formation using [6-$^{14}$C]-D-glucose. At 7 DIV, cortical neurons were incubated for 2 hours with medium containing labeled [6-$^{14}$C]-D-glucose (1 µCi/mmol). Perchloric acid (HClO$_4$) (250 µl, 3 M) was used to stop cellular metabolism and to lyse the cells. The release of intracellular CO$_2$ was captured by Whatman filter papers soaked in hyamine covering the lid over a period of 24 hours at room temperature. Next, the filter papers were transferred to scintillation vials and counted by the Scintillation Analyzer. OXIDATIVE PENTOSE PHOSPHATE PATHWAY (oxPPP) was measured as $^{14}$CO$_2$ formation using [6-$^{14}$C]-D-glucose ($^{14}$CO$_2$ formation only in the TCA cycle) and [1-$^{14}$C]-D-glucose ($^{14}$CO$_2$ formation both in the TCA cycle and the oxPPP). Cortical neurons were cultured in parallel wells and incubated with medium containing [6-$^{14}$C]-D-glucose (1 µCi/mmol) or medium containing [1-$^{14}$C]-D-glucose (1 µCi/mmol). Released $^{14}$CO$_2$ was captured in a similar manner as described above for glucose oxidation. The [6-$^{14}$C]-glucose flux was then subtracted from the [1-$^{14}$C]-glucose flux. The relative contribution of the oxPPP flux to glucose metabolism was assessed by including glycolytic flux in the relative flux measurements as described before (Ashcroft et al., 1972). GLUTAMINE OXIDATION was measured as $^{14}$CO$_2$ formation using [U-$^{14}$C]-glutamine. Cortical neurons were incubated with medium containing [U-$^{14}$C]-glutamine for 2 hours. Cells were lysed with HClO$_4$ and $^{14}$CO$_2$ was captured in hyamine-soaked filter papers. LACTATE OXIDATION was measured in a similar way as $^{14}$CO$_2$ formation after incubation with medium containing [U-$^{14}$C]-lactate. OXYGEN CONSUMPTION RATE (OCR) was measured using the extracellular flux analyzer XF24 (Seahorse Bioscience Inc.). Cortical neurons were plated and grown for 7 days in a Seahorse XF24 tissue culture plate. At 7 DIV, the oxygen consumption rate was measured over a period of 2 minutes. In baseline conditions, 5 consecutive measurements of OCR are done. Next, oligomycin, a blocker of ATP synthase is injected at 12 µM (concentration in well 1.2 µM). The drop in OCR reflects the oxygen consumption serving ATP production. In parallel wells, dinitrophenol (DNP), a mitochondrial uncoupler is injected at 1 mM (concentration in well 100 µM). This forces neurons to use their maximal mitochondrial capacity in order to preserve the mitochondrial membrane potential. The increase in OCR reflects the reserve capacity of mitochondria. As a second injection,
Antimycin-A is added at 10 µM (concentration in well 1 µM), a complex III inhibitor. This shuts down the electron transport chain activity abolishing the mitochondrial oxygen consumption, allowing the calculation of baseline mitochondrial respiration (OCR_{BAS}). The residual OCR corresponds to the non-mitochondrial oxygen consumption. All mitochondrial inhibitors were from Sigma. **HYPOXIA EXPOSURE:** To assess metabolic fluxes in hypoxia, we exposed neuronal cultures to 0.1 % O_2 during the 2 hours incubation with radiolabeled tracer glucose and glutamine.

**G6PD ACTIVITY MEASUREMENT**

G6PD activity was measured in neuronal lysates obtained from 7 DIV cultures, using the colorimetric Glucose 6 Phosphatase Assay Kit (Abcam) following manufacturer's instructions.

**ASSESSMENT OF REACTIVE OXYGEN SPECIES IN VITRO**

The scavenging capacity of reactive oxygen species (ROS) was measured by assessing the detoxification rate of H_2O_2 after loading the cells (7 DIV) with 10 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2-DCFDA, Molecular Probes, Invitrogen). CM-H_2-DCFDA is metabolized by intracellular esterases to a non-fluorescent product, which is oxidized to the fluorescent product CM-DCF, by H_2O_2. The dye was washed away and cells were exposed to 100 µM H_2O_2. Fluorescence was measured over time with a fluorescent plate reader. Mitochondria-localized superoxide radicals (O_2^-) were measured using the mitochondria-targeted, O2-sensitive fluorogenic probe mitoSOX Red (Molecular Probes, Invitrogen). Briefly, WT and PHD1^-/- neurons (7 DIV) were loaded with mitoSOX Red (5 µM) for 10 minutes at 37°C and 5% CO_2. Following fixation with 4 % PFA, mitoSOX Red fluorescence (Absorption/emission maxima: 510/580 nm) was detected using a Leica DMI6000B epifluorescence microscope (Leica, Manheim, Germany). Average fluorescent intensity and cell nuclei numbers were measured by Leica LASAF-MMAF morphometric analysis (Metamorph, Leica Microsystems, Mannheim, Germany).
**ASSESSMENT OF INTRACELLULAR SODIUM CONCENTRATION**

WT and PHD1⁻/⁻ neurons were cultured on coverslips and at 7-8 DIV loaded with 10 μM SBFI-AM (Invitrogen) and 0.06% Pluronic F-127 (Invitrogen) for 45 min at 37°C. The intracellular Na⁺ concentration ([Na⁺]ᵢ) was monitored using SBFI-based microfluorimetry. Changes in [Na⁺]ᵢ were monitored on an Olympus IX81 inverted microscope through the ratio (R) of fluorescence measured upon alternating illumination at 340 and 380 nm using an MT-10 illumination system and the xcellence pro software (Olympus, Planegg, Germany) at room temperature. To convert R to absolute [Na⁺]ᵢ values, an in situ calibration was performed as described earlier (Diarra et al., 2001). Briefly, cells were treated with 5 μM gramicidin D (Sigma), a channel-forming ionophore, and subsequently perfused with solutions containing 10 mM HEPES (pH 7.4), 1 mM MgCl₂, 2 mM CaCl₂ and increasing concentrations of NaCl (0, 5, 10, 50, 100, 150 mM), supplemented with KCl such that NaCl and KCl concentration always sums up to 150 mM. From these data, a calibration curve (R vs [Na⁺]) was constructed and fitted with the equation:

\[
[\text{Na}^+]_i = K_m \times \frac{(R-R_{\text{min}})}{(R_{\text{max}}-R)}.
\]

The deduced parameters (Kₘ, Rₘᵢₙ and Rₘₐₓ) were then used to calculate [Na⁺]ᵢ from experimental R values.

The standard extracellular solution for [Na⁺]ᵢ measurements contained 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose and 10 mM HEPES (pH 7.4). After equilibration for 10 minutes in this solution, R was monitored during 2 minutes, yielding a value for the basal [Na⁺]ᵢ. Subsequently, KCl was omitted from the standard extracellular solution during 10 minutes, thus inhibiting Na⁺ extrusion via Na⁺/K⁺-ATPases. This resulted in a robust rise in [Na⁺]ᵢ, which reflects basal Na⁺ influx via Na⁺-dependent transporters and ion channels. Finally, the cells were again perfused with the standard, K⁺-containing solution, resulting in a gradual restoration of the basal [Na⁺]ᵢ due to Na⁺ extrusion by the Na⁺/K⁺-ATPases.
**ANALYSIS OF PROTEIN AND RNA SYNTHESIS**

*Protein synthesis*: cortical neurons were labeled with 50 µM Click-IT L-Homopropargylglycine (HPG, Molecular Probes) for 24 hours to allow incorporation during protein synthesis. After fixation of the cells with 4% PFA, the incorporated HPG was detected by a ‘click reaction’ with Alexa-647 according to the manufacturer’s instructions.  

*RNA synthesis*: cortical neurons were labeled with 50 µM Click-IT ethinyl-uridine (EU, Molecular Probes) for 24 hours to allow incorporation during RNA synthesis. After fixation of the cells with 4% PFA, the incorporated EU was detected by a ‘click it reaction’ with Alexa-647 according to the manufacturer’s instructions. HPG or EU signal intensities and cell nuclei numbers were measured by Leica LASAF-MMAF morphometric analysis (Metamorph, Leica Microsystems, Mannheim, Germany).

**LC-MS ANALYSIS OF GLUTATHIONE (GSH-GSSG ASSAY)**

Cultured neurons were extracted in 100 µl 5% TCA and cortex tissue from stroke area and corresponding contralateral area were extracted in 500 µl 5% TCA. Following extraction, the lysate was transferred to a fresh Eppendorf tube and 100 µl mq H2O was added and the mixture centrifuged for 10 minutes at 20,000 x g. The supernatant was transferred to a fresh vial and 100 µl, was injected onto an Acquity UPLC HSS T3 column (2.1 x 100 mm, particle size 1.8 µm, cat # 186003539, Waters) using an Ultimate 3000 UPLC (Thermo Scientific) in-line connected to a Q-Exactive OrbiTRAP mass spectrometer (Thermo Scientific). The column was thermostated at 37 °C. A linear gradient was carried out using solvent A (0.05% formic acid) and solvent B (60% methanol, 0.05% formic acid). Briefly, 1% solvent B was maintained for 10 minutes, then increased to 100% B at 12 minutes and kept at 100 % B for 3 minutes (t =15 minutes). The gradient was returned to 1% solvent B at 16 minutes and allowed to re-equilibrate until 21 minutes. The flow rate was kept constant at 250 µl/minute. Elution of GSH and GSSG was observed at respectively 3 and 6 minutes. The mass spec operated in targeted SIM mode following ions with m/z 308.09108 (GSH, z=1) and 613.15979 (GSSG, z=1) eluting between 1 and 10 minutes of the gradient. The mass spectrometer was set to positive ion mode, AGC target of 1 e6
ions and a maximum injection time of 50 ms. The sheath gas flow was set at 35, the auxiliary gas flow rate at 10. The spray voltage was used at 3.00 kV and the capillary was heated at 350 °C. Auxiliary gas heater temperature was placed at 300 °C.

**GC-MS analysis of glucose consumption**

At 5 DIV, culture medium of WT and PHD1<sup>−/−</sup> neurons was changed to equal amounts of fresh medium. Twenty four hours later, medium samples were collected. Glucose content was assessed by MS. Glucose consumption was determined as the difference between blank samples (medium 24 hours without cells) and the medium obtained from WT and PHD1<sup>−/−</sup> neuronal cultures. Values were corrected by cell number (protein content). To 50 µl of medium 450 µl of methanol (Ultrapure, Sigma-Aldrich) at -80°C was added. The solution was put for 2 hours at -80°C and supernatant was collected following a centrifugation step at 4°C for 15 min at 20,000 x g. Next, the supernatant was dried using a vacuum centrifuge. To the dried fractions 50 µl of hydroxylamine hydrochloride solution (Sigma-Aldrich, Bornem, Belgium) was added, the reaction was carried out for 60 min at 90°C, samples were cooled down and 100 uL of propionic anhydride (Sigma) was added. The reaction lasted for 30 min at 60°C. Next, samples were dried and fractions were dissolved in 100 uL ethyl acetate and transferred to appropriate Agilent glass vials with conical inserts. GC-MS analyses were performed using an Agilent 7890A GC equipped with a HP-5 ms 5% Phenyl Methyl Silox (30 m - 0.25 mm i.d. - 0.25 µm; Agilent Technologies, Santa Clara, California, USA) capillary column, interfaced with a triple quadrupole tandem mass spectrometer (Agilent 7000B, Agilent Technologies) operating under ionization by electron impact at 70 eV. The injection port, interface and ion source temperatures were kept at 230 °C. Temperature of the quadrupoles was maintained at 150°C. The injection volume was 1 µl, and samples were injected at 1:75 split ratio. Helium flow was kept constant at 1 ml/min. The temperature of the column started at 80 °C for 1 min and increased to 280 °C at 20 °C/min, and held for 3 min. Next, column regeneration was carried out for 3 min at 325°C. The GC/MS/MS analyses were performed in Single Ion Monitoring (SIM) scanning for the m/z 370,15.
**LC-MS ANALYSIS OF GLYCOLYTIC INTERMEDIATES**

The polar metabolites were extracted using a cold two-phase methanol-water-chloroform extraction (Fendt et al., 2013; Schoors et al., 2015). The dried metabolite samples were resuspended in 60% acetonitrile and transferred to LC-MS vials. Measurements were performed using a Dionex UltiMate 3000 LC System (Thermo Scientific) in-line connected to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). 15 ul of sample was injected and loaded onto a SeQuant ZIC/ pHILIC Polymeric column (Merck Millipore). A linear gradient was carried out starting with 84% solvent A (95-5 acetonitrile-H2O, 2mM ammoniumacetate pH 9.3) and 16% solvent B (2 mM ammoniumacetate pH 9.3). From 2 to 29 minutes the gradient changed to 75% B and was kept at 75% until 34 min. Next a decrease to 16% B was carried out to 42min and then 16% B was maintained until 58 min. The solvent was used at a flow rate of 100 ul/min, the columns temperature was kept constant at 25 degrees Celsius. The mass spectrometer operated in negative ion mode, settings of the HESI probe were as follows: sheath gas flow rate at 25, auxiliary gas flow rate at 5 (at a temperature of 260 degrees Celsius). Spray voltage was set at 4.8 kV, temperature of the capillary at 300 degrees Celsius and S-lens RF level at 50. Both a full scan (resolution of 35.000 and scan range of m/z 50-1050) as well as a targeted SIM method focusing on ions from glycolytic intermediates was applied. Data collection (abundance and 13C isotopic incorporation) was performed using Xcalibur software (Thermo Scientific).

**HPLC ANALYSIS OF ENERGY CHARGE**

Cells were harvested in 100 µl ice cold 0.4 M perchloric acid supplemented with 0.5 mM EDTA. pH was adjusted by adding 100 µl of 2 M K2CO3. One hundred µl of the mixture was injected onto an Agilent 1260 HPLC equipped with a C18-Symmetry column (150 x 4.6 mm; 5 mm) (Waters), thermostated at 22.5 °C. Flow rate was kept constant at 1 ml/min. A linear gradient using solvent A (50 mM NaH2PO4, 4 mM tetrabutylammonium, adjusted to pH 5.0 using H2SO4) and solvent B (50 mM NaH2PO4, 4 mM
tetrabutylammonium, 30% CH$_3$CN, adjusted to pH 5.0 using H$_2$SO$_4$) was accomplished as follows: 95% A for 2 minutes, from 2 to 25 minutes linear increase to 100% B, from 25 to 27 minutes isocratic at 100% B, from 27 to 29 minutes linear gradient to 95% A and finally from 29 to 35 minutes at 95% A. Detection of ATP, ADP and AMP occurred at 259 nm. The energy charge is calculated as ([ATP] + ½ [ADP] / [ATP] + [ADP] + [AMP]).

**IMMUNOCYTOCHEMISTRY, HISTOCHEMISTRY AND MORPHOMETRIC ANALYSIS**

Cortical neurons grown on Willco-Dish glass bottom dishes (Ibidi) were fixed after 7 days in culture with PFA 4% for 10 minutes. After permeabilization for 10 minutes in 0.25% Triton and blocking 30 for minutes with 10% pre-immune serum, the cells were incubated overnight with the appropriate primary antibodies. The following antibodies were used: anti-TOMM20 (ab56783, Abcam; 1/200), anti-MAP2 (M3696, Sigma, 1/500), anti-GFAP (G3893, Sigma, 1/500), anti-NG2 (AB5320, Chemicon, 1/200), anti-Olig2 (AB9610, Chemicon, 1/20,000). The next day, the cultures were washed and incubated with the appropriate fluorescently conjugated secondary antibody (Alexa-568 or Alexa-488, Molecular Probes, Invitrogen). Fluorescence imaging was performed using Zeiss LSM 780 confocal microscope (Zeiss, Germany) or Leica DMI6000B epifluorescence microscope (Leica, Germany). Signal positive areas were quantified using the FIJI / NIH Image J software package / AxioVision morphometric analysis software (Carl Zeiss, Munich, Germany) with in-house developed macros. Quantification of arborization of tdT-transfected neurons was performed by the module for Sholl analysis in FIJI software. Nissl staining on brain sections was performed using standard protocols. Bright fields imaging was performed using a Leica DMI6000B epifluorescence microscope (Leica, Germany). Quantification of cortical thickness and lateral extension were performed using FIJI software. All quantifications were performed by researchers blinded for the genotype.

**RNA, DNA AND PROTEIN ANALYSIS**

**RNA LEVEL ANALYSIS:** Total RNA was extracted from cultured cortical neurons using the RNeasy microkit (Qiagen), and reverse transcribed using the Quantitect Reverse
Transcription kit (Qiagen) according to the manufacturer’s instructions. Total RNA was extracted from dissected brain tissue using Trizol-chloroform extraction. After tissue homogenization with Trizol, chloroform was added to separate the aqueous phase. After centrifugation and addition of ethanol, samples underwent a RNA clean-up on a RNeasy mini column (Qiagen 74104) with DNase I treatment (Invitrogen 12185010). Gene expression analysis was performed by Taqman quantitative RT-PCR, using premade primer sets (Applied Biosystems or IDT). Expression levels were quantified relative to the expression level of housekeeping genes (β-actin or HPRT). Primers and probes ID numbers are available upon request. **MITOCHONDRIAL DNA CONTENT:** For mitochondrial DNA copy measurement, total DNA was extracted from cell lysates. The amount of mitochondrial DNA relative to nuclear genomic DNA was determined by quantitative PCR using homemade primers for the genic sequences of cytochrome b (mitochondrial, MIT) and RPL13A (nuclear, NC). Forward, reverse and probe sequences were: NC FOR2 5’-aga cct gaa gag gct cta a-3’; NC REV2 5’-cag ctt ggc gat cca gct-3’; NC probe 5’-FAM-cca ccc tcc ctt cca gct acc atc cta-TAMRA-3’; and MIT FOR 5’-ttg atc aac gga cca agt tac c-3’; MIT REV 5’-cgt tga aca aac gaa cca tta ata g-3’; MIT probe 5’-FAM-aca att agg gtt tac gac ctc gat gtt gga t-TAMRA-3’. **PROTEIN EXPRESSION (WESTERN BLOT ANALYSIS):** Protein extraction and immunoblot analysis were performed using a modified Laemli sample buffer (125 mM Tris-HCl, pH 6.8 buffer containing 2% SDS and 10% glycerol) in the presence of protease and phosphatase inhibitors (Roche). Nuclear protein extraction was done by using the NEPER kit according to manufacturer’s instructions. Lysates were separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane (Life Technologies), and analyzed by immunoblotting. Primary antibodies used were goat anti-HIF1α (AF1935, R&D Systems, 1:200), goat anti-HIF2α (AF2997, R&D Systems, 1:200), rabbit anti-PHD2 (NB100-2219, Abcam, 1:1000), rabbit anti-PHD3 (1088-100, Abcam, 1:1000), rabbit anti-p65 (No. 3033 Cellsignaling, 1:1000). Equal loading was verified by incubation with rabbit anti-β-actin (13E5; No. 4970, Cell signaling), mouse anti-α-tubulin (Sigma, 1:10000) or rabbit anti-lamin A/C (No. 2032, Cell Signaling). Appropriate secondary antibodies were HRP-conjugated (Dako or Cell signaling). Signal was detected
using the ECL system (Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions.

**IN VIVO INFUSION OF 1,2-\(^{13}\)C GLUCOSE**

**SURGICAL CATHETERIZATION OF THE RIGHT JUGULAR VEIN:** Mice were anesthetized with 1% isoflurane and a catheter was placed into the right jugular vein. A small incision between the shoulder blades was made and the catheter was exteriorized at the back of the mouse and connected to an antenna part, which enables conscious handling of the mouse during infusion. The tubing was flushed with heparinized saline directly after the surgery and 3 days later (Ayala et al., 2006). Animals were individually housed after surgery, and painkillers were administered (Carprofen, 5 mg/kg body weight subcutaneous) during surgery and one day after. **INFUSION WITH \(^{13}\)C LABELED GLUCOSE AND TISSUE PROCESSING:** Five days after surgery, [1,2-\(^{13}\)C]-labeled glucose (99% enriched; Buchem) was infused in awake, freely moving mice. Morning fasted mice (6 hours) were subjected to a continuous infusion of 0.03 mg \(^{13}\)C labeled glucose per gram body weight per minute during 6 hours (yielding a final \(^{13}\)C plasma glucose enrichment of 64 ± 12%). Subsequently, the mice were sacrificed by cervical dislocation, blood was collected and different brain regions were dissected within less than 5 minutes and immediately frozen using a liquid nitrogen cooled Biossqueezzer (Biospec Products). The tissue was weighed (10-15 mg) and pulverized (Cryomill, Retsch) under liquid nitrogen conditions. The polar metabolites were extracted using a cold two-phase methanol-water-chloroform extraction (Fendt et al., 2013; Schoors et al., 2015). The dried metabolite samples were resuspended in 60% acetonitrile and transferred to LC-MS vials. Metabolite measurements were performed using a Dionex UltiMate 3000 LC System (Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) operated in negative mode. Fifteen µl of sample was injected on a SeQuant ZIC/ pHILIC Polymeric column (Merck Millipore). The solvent was used at a flow rate of of 100 µl/min and was composed of an acetonitrile / ammonium acetate (pH 9.3, 2 mM) mixture. Data collection was performed using Xcalibur software (Thermo Scientific). Blood glucose enrichment in the infused mice was measured as
described above in the section on GC-MS analysis of glucose consumption. Pilot experiments in cultured neurons incubated with [1,2-¹³C]-glucose showed that the percentage of M+1 labeling in lactate and alanine, which is commonly used to reflect the PPP activity, was not affected by PHD1 deficiency. Nevertheless, PHD1⁻/⁻ neurons show enhanced flux as measured by the differential ¹⁴CO₂ production from [1-¹⁴C]-glucose and [6-¹⁴C]-glucose, which reflects the oxidative branch of the PPP. These data suggest that the enhanced flux through the oxidative branch does not result in a subsequent increase of the non-oxidative PPP flux towards glycolysis. We therefore quantified the ratio of the fractional M+1 vs M+2 labeling of pentose-5-phosphate (P5P), as this is indicative for the relative importance of the oxPPP vs non-oxPPP. While also M+4 can arise from a net of exchange flux of the non-oxidative PPP towards P5P, the M+4 enrichment was substantially lower than the M+2 enrichment. We therefore considered only the M+1 and M+2 enrichment in P5P to characterized the relative importance of the oxidative vs non-oxidative PPP.

**ICV DELIVERY OF ANTISENSE OLIGONUCLEOTIDES AGAINST PHD1 IN STROKE**

Oligonucleotides directed against murine PHD1 were designed and purified by Isis Pharmaceuticals. These antisense oligonucleotides (ASOs) are directed against the regions within the coding region and 5’ and 3’ untranslated region of the PHD1 mRNA. After binding to complementary target RNA through Watson-Crick base-pairing, the target RNA will be degraded by RNaseH-mediated RNA degradation. Additional modifications consisting of a O-(2-methoxy)ethyl substitution at the 2’ position and phosphorothioate substitution were created to increase the resistance of the ASOs against nuclease activity and in this way promote their stability. Three top ASOs of 20 nucleotides long were selected for further investigation. Knockdown efficiency was tested in cultured cortical neurons. At 2 DIV, cortical neurons were exposed to 0.5 µM and 1 µM of anti-PHD1 ASOs. To ensure correct dosing, the absorbance at 260 nm was regularly measured in the stock solution. An oligonucleotide that targets murine MALAT-1 (Metastasis associated lung adenocarcinoma transcript; non coding RNA in the nucleus) was used as control ASO.
Similar to MALAT-1 knockout mice (Zhang et al., 2012), anti-MALAT1 ASO does not induce a phenotype in the central nervous system and is well tolerated in neurons (C.F.B., G.H., unpublished data). At 7 DIV, cortical neurons were harvested for RNA extraction and qPCR for PHD1, PHD2 and PHD3.

One out of the three ASOs with the highest efficiency and specificity was selected for further in vivo studies. A dose of 75 µg ASO/day (selected in pilot experiments) was infused into the left lateral ventricle of male C57Bl/6N mice (age 7-8 weeks), using Alzet® osmotic pumps (model 2004, Cupertino), connected with a catheter to a brain infusion cannula. The brain infusion assembly was filled with a solution, containing the ASO diluted in artificial CSF (aCSF) or with aCSF alone (saline control). For the in vivo studies both saline and a non-RNAase H active ASO to human huntingtin was used as control. After anesthesia with ketamine and xylazine, the osmotic pump was subcutaneously inserted in the midscapular area on the back of the mouse. After exposing the skull through a midline sagittal incision, a hole was drilled through the skull, and the cannula placed using the following stereotactic coordinates: 0 mm posterior and 1 mm lateral to bregma, and 2 mm deep from skull surface. After 10 days of infusion, brains were harvested from a series of test mice to assess knockdown efficiency. Two weeks after the infusion, pMCAO was performed. There was no difference in stroke size between saline and control ASO groups, demonstrating that injection of an ASO per se did not cause additional effects that would influence stroke outcome.
SUPPLEMENTAL REFERENCES


