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RED CELLS, IRON, AND ERYTHROPOIESIS

Prolyl-4-hydroxylase 2 and 3 coregulate murine erythropoietin in brain pericytes

Andres A. Urrutia,[1] Aqeela Afzal,[1,2] Jacob Nelson,[1] Olena Davidoff,[1,3] Kenneth W. Gross,[4] and Volker H. Haase[1,3,5]

[1]Department of Medicine and [2]Department of Neurosurgery, Vanderbilt University Medical Center, Nashville, TN; [3]Medical and Research Services, Department of Veterans Affairs Hospital, Tennessee Valley Healthcare System, Nashville, TN; [4]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY; and [5]Departments of Cancer Biology and Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN

Key Points

- Pericytes function as oxygen sensors and are major sites of erythropoietin production in the hypoxic brain.
- The ability to synthesize erythropoietin is a functional feature of pericytes in the brain and kidney.

A classic response to systemic hypoxia is the increased production of red blood cells due to hypoxia-inducible factor (HIF)-mediated induction of erythropoietin (EPO). EPO is a glycoprotein hormone that is essential for normal erythropoiesis and is predominantly synthesized by peritubular renal interstitial fibroblast-like cells, which express cellular markers characteristic of neuronal cells and pericytes. To investigate whether the ability to synthesize EPO is a general functional feature of pericytes, we used conditional gene targeting to examine the von Hippel-Lindau/prolyl-4-hydroxylase domain (PHD)/HIF axis in cell-expressing neural glial antigen 2, a known molecular marker of pericytes in multiple organs. We found that pericytes in the brain synthesized EPO in mice with genetic HIF activation and were capable of responding to systemic hypoxia with the induction of *Epo*. Using high-resolution multiplex in situ hybridization, we determined that brain pericytes represent an important cellular source of *Epo* in the hypoxic brain (up to 70% of all *Epo*-expressing cells). We furthermore determined that *Epo* transcription in brain pericytes was HIF-2 dependent and cocontrolled by PHD2 and PHD3, oxygen- and 2-oxoglutarate–dependent prolyl-4-hydroxylases that regulate HIF activity. In summary, our studies provide experimental evidence that pericytes in the brain have the ability to function as oxygen sensors and respond to hypoxia with EPO synthesis. Our findings furthermore suggest that the ability to synthesize EPO may represent a functional feature of pericytes in the brain and kidney. (*Blood*. 2016;128(21):2550-2560)

Introduction

A classic response to hypoxia is the rise in red blood cell (RBC) numbers, which increases the oxygen-carrying capacity of blood and thus improves tissue oxygenation.[1] This prototypical hypoxia response is mediated by the glycoprotein hormone erythropoietin (EPO), which is mainly produced in kidney and liver and induces erythropoiesis by preventing apoptosis of erythroid precursor cells.[2]

In the kidney, the main source of EPO in adults, a small number of erythropoietin-producing cells (EPCs) is found in the cortico-medullary region at baseline, whereas under hypoxic conditions, renal EPCs increase in number and expand spatially toward the outer cortex.[2] Renal EPCs are derived from forkhead box D1 (FOXD1) stroma and express a variety of cellular markers characteristic of pericytes and neuronal cells.[3,4] These include platelet derived growth factor receptor-β polypeptide (PDGFRB) and chondroitin sulfate proteoglycan 4 (CSPG4), also known as high-molecular-weight melanoma-associated antigen or neuro-glial antigen 2 (NG2), as well as microtubule-associated protein 2 (MAP-2) and neurofilament protein light polypeptide (NF-L).[3-5] Although the kidney is the main site of adult EPO synthesis, systemic hypoxia, anemia, or genetic defects in the hypoxia-inducible factor (HIF) pathway trigger EPO production in other tissues such as liver.[6-10] Research into nonrenal sites of EPO production has also identified neurons and astrocytes as sources of EPO.[6,11-13] However, the role and relative contribution of these and other cerebral cell types to the brain's EPO response has not been defined.

Pericytes or vascular mural cells are perivascular cells that wrap around endothelial cells, embedd within the endothelial basement membrane, and thus form an integral part of the tissue's microvasculature. In areas lacking a basement membrane, the cell membranes of pericytes and endothelial cells form peg-and-socket contacts allowing direct communication and exchange of molecules.[14] Pericytes can be identified by their expression of PDGFRB, α-smooth muscle actin (αSMA), and/or NG2.[15-18] Developmentally, cell fate tracing studies have suggested that pericytes in kidney and forebrain derive from the neural crest,[5,19] whereas pericytes in other organs, such as liver, lung, heart, or gut are mesothelium derived.[20-23]

The hypoxic induction of EPO in kidney and liver is regulated by HIF-2, a heterodimeric basic helix-loop-helix (bHLH) transcription factor that consists of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit.[24-26] To date, 3 different HIF-α-subunits have been identified: HIF-1α, HIF-2α, and HIF-3α.[27-29] Although continuously synthesized, HIF-α subunits are rapidly degraded in the presence of molecular oxygen. HIF degradation is controlled by oxygen-, iron-, and ascorbate-dependent

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prolyl-4-hydroxylase domain-containing proteins (PHD)-1, -2, and -3, also known as EGLN2, EGLN1, and EGLN3, respectively, which use 2-oxoglutarate (OG) as substrate for the hydroxylation of specific proline residues within HIF-α.[30-33] This leads to binding to the von Hippel-Lindau (VHL)–E3 ubiquitin ligase complex and subsequent degradation by the proteasome. When oxygen levels decrease, HIF proline-4-hydroxylation is reduced, and HIF-α subunits are no longer degraded, translocate to the nucleus, and hetero-dimerize with HIF-β, promoting the transcription of oxygen-regulated genes.[27]

Because of their functional relevance for vascular homeostasis and the molecular features shared with renal EPCs, we investigated the HIF-EPO axis in pericytes and targeted the main components of the HIF oxygen-sensing pathway in NG2-expressing cells. Here we report that pericytes in the brain responded to *Vhl* inactivation with an increase in *Epo* mRNA transcription. Using multiplex high-resolution RNA fluorescent in situ hybridization (FISH), we identified brain pericytes as major contributors to the cerebral EPO response to hypoxia. We furthermore establish that *Epo* transcription in NG2 cells is controlled by both PHD2 and PHD3, as only the combined inactivation of *Phd2* and *Phd3* led to an increase in brain *Epo,* which was completely dependent on HIF-2 activity. Thus, our studies identify pericytes as major cellular sources of EPO in the brain and suggest that the ability to synthesize EPO represents a functional feature of pericytes in brain and kidney.

Materials and methods

Generation and genotyping of mice and animal procedures

The generation and genotyping of mice carrying *Ng2-cre* and floxed alleles for *Vhl*, *Hif1a*, *Hif2a, Epo, Phd1* (*Egln2*), *Phd2* (*Egln1*), and *Phd3 (Egln3)* has been described elsewhere.[15,25,34-36] All procedures involving mice were performed in accordance with National Institutes of Health guidelines for the use and care of live animals and were approved by Vanderbilt University's Institutional Animal Care and Use Committee. A detailed description of mouse studies can be found in supplemental Materials and Methods available on the *Blood* Web site.

DNA and RNA analysis

DNA analysis for genotyping was performed as described previously.[36,37] RNA was isolated using the RNeasy kit according to manufacturer's protocol (Qiagen, Valencia, CA). For real-time polymerase chain reaction (PCR) analysis, mRNA expression levels were quantified with the relative standard curve method according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Further details can be found in supplemental Materials and Methods.

Immunofluorescence, RNA FISH, and immunoglobulin G extravasation studies

Detailed methodologic information can be found in supplemental Materials and Methods.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed with Prism 5 software (GraphPad Software, La Jolla, CA) using the unpaired 2-tailed Student *t* test or 1-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis to compare between 3 or more groups. $P < .05$ was considered statistically significant.

Results

Vhl inactivation in NG2 cells results in polycythemia

To investigate the HIF/EPO axis in NG2 pericytes, we first activated HIF signaling by ablating the VHL tumor suppressor as proof-of-concept study. For this, we crossed female mice that expressed Cre-recombinase under the control of the *Cspg-4/Ng2* promoter to mice homozygous for the *Vhl* floxed allele generating *Ng2-cre* $Vhl^{f/f}$ mutants, from hereon referred to as NG2-$Vhl^{-/-}$ mice. NG2 is a 300-kDa single membrane spanning chondroitin sulfate proteoglycan that acts as a coreceptor for platelet-derived growth factor (PDGF) and regulates cell proliferation and motility.[38] Proteoglycan NG2 is expressed in vascular mural cells/pericytes, mesenchymal lineage cells such as chondrocytes, osteoblasts, myoblasts, and skin stem cells, and in polydendrocytes, which are CNS precursor cells that give rise to oligodendrocytes and gray matter protoplasmic astrocytes.[18,39,40]

NG2-$Vhl^{-/-}$ mutant mice developed erythematous paws, ears, and muzzle and splenomegaly (Figure 1A). This was associated with elevated hematocrit (Hct), a significant increase in RBC numbers, hemoglobin (Hb), and plasma EPO concentration (Figure 1B). Despite severe polycythemia, NG2-$Vhl^{-/-}$ mice were viable and fertile. In contrast to NG2-$Vhl^{-/-}$ mice, mice with heterozygous *Vhl* deficiency (NG2-$Vhl^{+/-}$) did not develop polycythemia and were characterized by normal serum EPO levels (Figure 1B).

Vhl inactivation in NG2 cells increases brain EPO production

Because NG2-$Vhl^{-/-}$ mice were characterized by elevated plasma EPO concentration, we asked whether this was a result of increased EPO synthesis in multiple organs[41] and examined *Epo* transcript levels in different tissues. Whereas statistically significant differences in *Epo* expression were not detected in liver, heart, retina, spleen, muscle, and skin, *Epo* mRNA levels in brain, kidney, and bone were elevated compared with Cre^{-} control, with the brain displaying the most pronounced increase (~70-fold; Figure 1C). This rise in renal and brain *Epo* mRNA levels was associated with a ~2-fold (6.5 ± 0.25 vs 15 ± 2.5 pg/mg total kidney tissue protein) and ~40-fold (14.8 ± 1.1 vs 593 ± 99.1 pg/mg total brain tissue protein) increase in whole kidney and brain EPO protein levels (Figure 1C). These findings suggested that kidney, brain, and bone had contributed to the elevation in plasma EPO in NG2-$Vhl^{-/-}$ mice, however, the degree of contribution from individual tissues is unclear and difficult to assess with currently available technology.

To examine regional differences in cerebral *Epo* transcription, we dissected the brain and analyzed *Epo* expression levels in cortex, striatum, hypothalamus, and hippocampus by real-time PCR. Whereas NG2-$Vhl^{+/-}$ mice did not differ from their littermate controls, *Epo* levels in NG2-$Vhl^{-/-}$ mice were increased in all subregions, with hypothalamus displaying the highest level of increase (~50-fold), followed by striatum (~30-fold), and then cortex and hippocampus (~15-fold each; Figure 1D).

To assess whether increased brain *Epo* expression resulted directly from enhanced *Epo* transcription in $Vhl^{-/-}$ cells or nontargeted adjacent cells, we generated NG2-$Vhl^{-/-}Epo^{-/-}$ double mutant mice. Conditional coinactivation of *Vhl* and *Epo* in NG2 cells reduced brain *Epo* mRNA levels to control levels in cortex, striatum, and hippocampus, whereas *Epo* mRNA levels in hypothalamus were lower than in controls (Figure 2B). The normalization of brain *Epo* mRNA expression was furthermore associated with normal RBC parameters

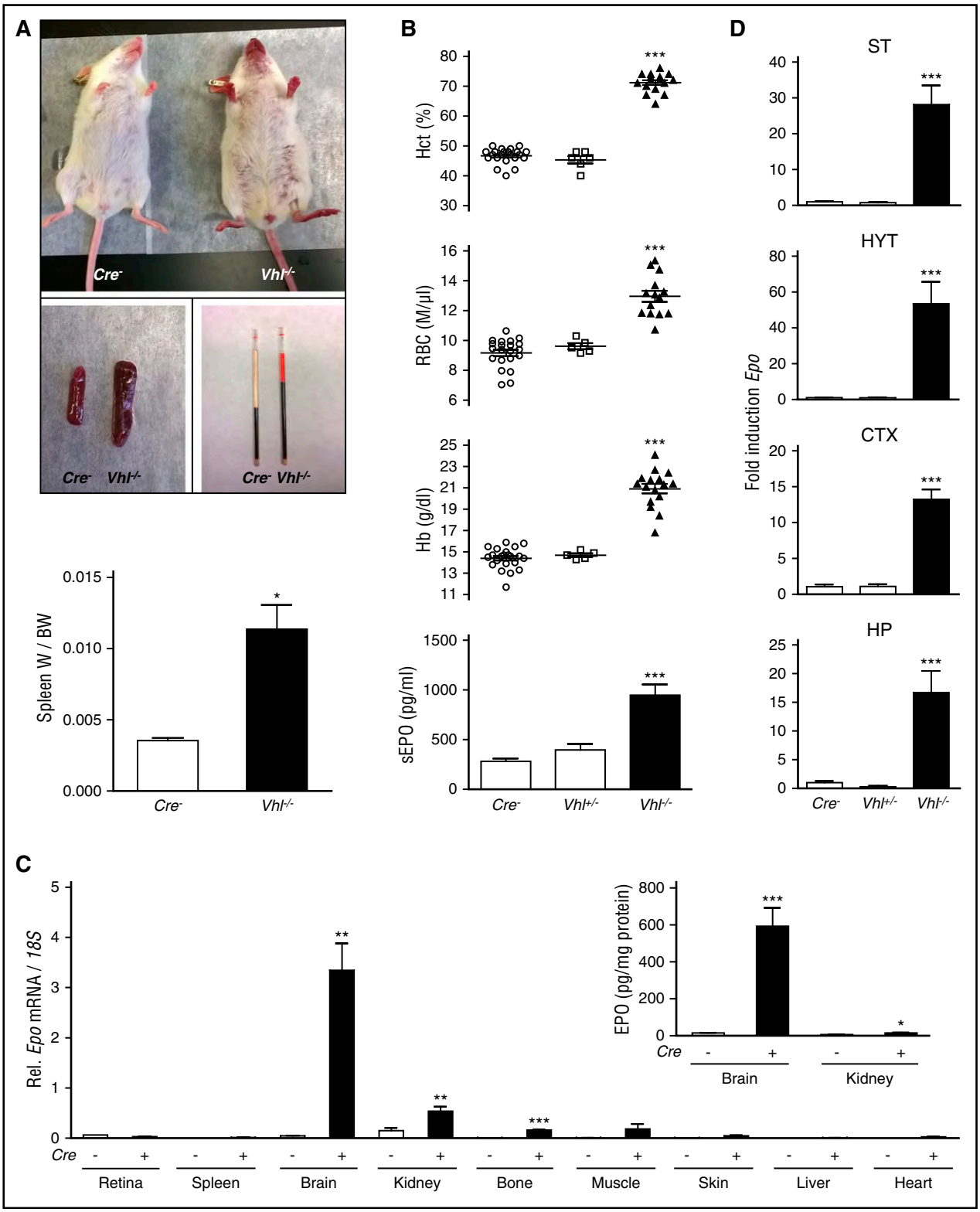

Figure 1. NG2 cell-specific inactivation of *Vhl* results in polycythemia. (A) Mice with NG2 cell-specific inactivation of *Vhl* develop red paws, ears, and snout, splenomegaly, and polycythemia (n = 3-7). (B) Inactivation of *Vhl* in NG2 cells induces erythropoiesis and increases plasma EPO. Shown are individual Hct, RBC, and Hb values for Cre^- control (n = 22), NG2-$Vhl^{+/-}$ (n = 6), and NG2-$Vhl^{-/-}$ mice (n = 16). Bar graphs show serum EPO levels (sEPO) for Cre^- control (n = 20), NG2-$Vhl^{+/-}$ (n = 6), and NG2-$Vhl^{-/-}$ (n = 11) mice. Data are represented as mean ± SEM; 1-way ANOVA followed by Tukey's post hoc analysis; ***$P < .001$ compared with control group. (C) Relative *Epo* mRNA levels in retina, spleen, brain, kidney, bone, muscle, skin, liver, and heart in Cre^- control and NG2-$Vhl^{-/-}$ mice (n = 3-7). (Inset) Brain and kidney EPO protein levels in Cre^- control and NG2-$Vhl^{-/-}$ mice expressed as pg/mg total tissue protein (n = 5-7). Data are represented as mean ± SEM; 2-tailed Student *t* test; *$P < .05$, **$P < .01$, and ***$P < .001$ compared with Cre^- controls. (D) *Epo* mRNA levels in striatal (ST), hypothalamic (HYT), cortical (CTX), and hippocampal (HP) subregions from Cre^- (n = 12), NG2-$Vhl^{+/-}$ (n = 3), and NG2-$Vhl^{-/-}$ mice (n = 4). Data are represented as mean ± SEM; 1-way ANOVA followed by Tukey's post hoc analysis; ***$P < .001$ compared with the Cre^- control group. BW, total body weight; W, organ weight.

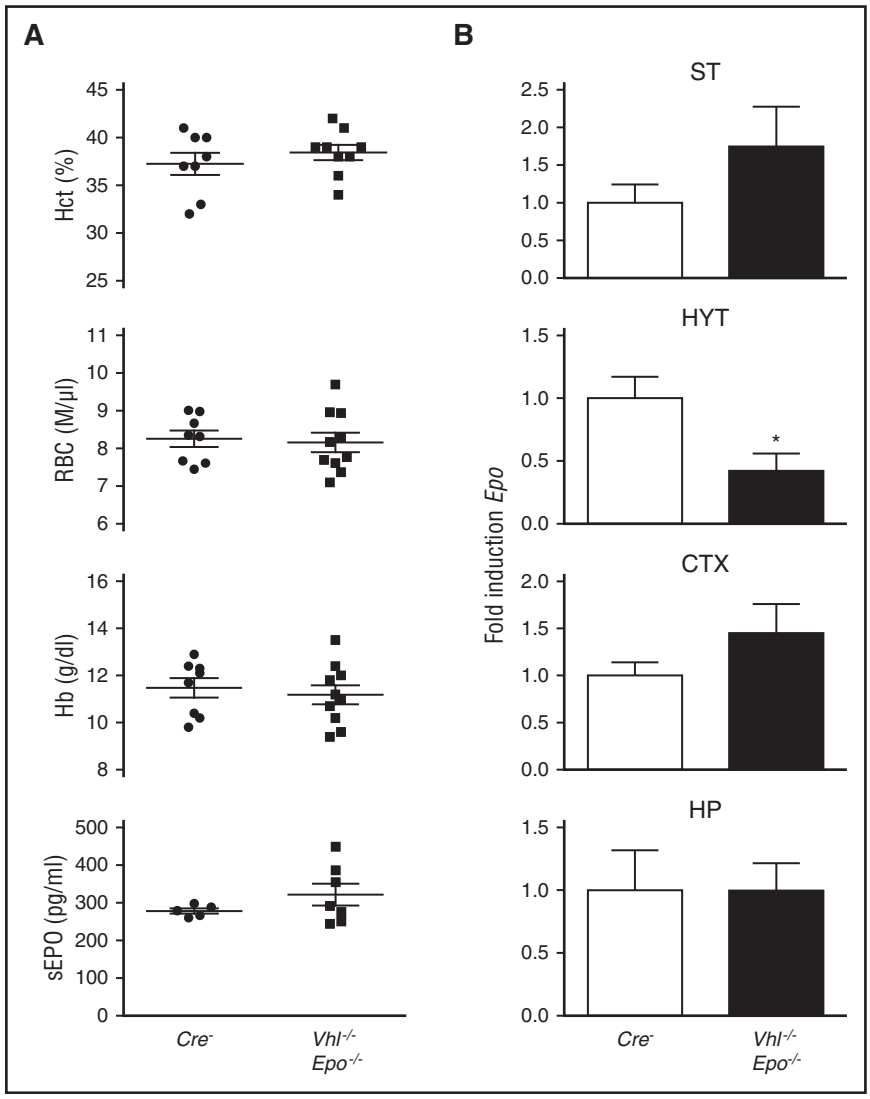

Figure 2. *Epo* induction in NG2-*Vhl*$^{-/-}$ mice is cell autonomous. (A) Shown are individual Hcts, RBC, and Hb values for Cre^{-} control and in NG2-*Vhl*$^{-/-}$*Epo*$^{-/-}$ mice (n = 8-10). Bar graphs show serum EPO levels (sEPO) for Cre^{-} and NG2-*Vhl*$^{-/-}$*Epo*$^{-/-}$ mice (n = 5-7). (B) Striatal (ST), hypothalamic (HYT), cortical (CTX), and hippocampal (HP) *Epo* mRNA levels in Cre^{-} and NG2-*Vhl*$^{-/-}$*Epo*$^{-/-}$ mice (n = 4 each). Data are represented as mean ± SEM; 2-tailed Student *t* test; **P* < .05 compared with Cre^{-} control group.

and serum EPO levels, demonstrating that polycythemia in NG2-*Vhl*$^{-/-}$ mice was a cell-autonomous phenotype that resulted directly from the deletion of *Vhl* in NG2-expressing cells (Figure 2A). The absence of polycythemia in *NG2-Vhl*$^{-/-}$*Epo*$^{-/-}$ animals furthermore indicated that HIF activation in non–EPO-producing NG2 cells outside the brain, eg, in the bone marrow perivascular niche, had not contributed to erythropoiesis in these mice.[42] Taken together, our data suggest that the induction of *Epo* in NG2-expressing cells and their derivatives was cell autonomous.

Brain pericytes synthesize EPO in NG2-Vhl$^{-/-}$ mice

To assess the cellular source of *Epo* in NG2-*Vhl*$^{-/-}$ mice, we first examined to what degree pericytes were targeted by *Ng2-cre*. For this, we used a green fluorescence protein (GFP) Cre-reporter transgenic line, *ROSA26-ACTBtdTomato,-EGFP*, where EGFP is only expressed in cells that have a history of Cre-recombinase expression and have undergone Cre-loxP–mediated recombination. This Cre-reporter is under the control of the *Rosa26* promoter. Because NG2-*mT/mEGFP-Vhl*$^{-/-}$ mice cannot be generated due to the close proximity of the *Rosa26* and *Vhl* gene loci, we used mice for the analysis that were mutant for *Phd2*. Although Cre^{-} littermate controls did not exhibit EGFP expression, reporter mice displayed widespread EGFP expression in the brain that colocalized with ~35% of the PDGFRB-expressing cells (Figure 3).

Because NG2 is expressed in pericytes, and also polydendrocytes, which give rise to oligodendrocytes and gray matter astrocytes,[40,43] we next determined the exact cellular localization of *Epo* transcripts in brains from NG2-*Vhl*$^{-/-}$ mice by performing high-resolution RNA FISH with probes specific for the *Epo* and pericyte marker *Pdgfrb*.[16-18,44,45] We then quantified *Epo*- and *Pdgfrb*-expressing cells and determined the proportion of *Epo*$^{+}$*Pdgfrb*$^{+}$ cells among *Epo*- or *Pdgfrb*-expressing cells in cortex, striatum, hippocampus, and hypothalamus, as well as in the corpus callosum, where nonpericyte NG2 lineage cells are known to be more abundant. Although only 25% of all *Pdgfrb*$^{+}$ cells expressed *Epo*, we found that the majority of *Epo*$^{+}$ cells coexpressed *Pdgfrb* (~60%) in all brain subregions. *Epo*$^{+}$*Pdgfrb*$^{-}$ cells were found in vascular/perivascular regions and brain parenchyma (Figure 4). Taken together, our data indicate that pericytes are the main cellular source of *Epo* in the brain of NG2-*Vhl*$^{-/-}$ mice.

Hypoxia induces EPO production in brain pericytes

The analysis of NG2-*Vhl*$^{-/-}$ mice suggested a role for pericytes in cerebral EPO production. We next sought to examine whether brain

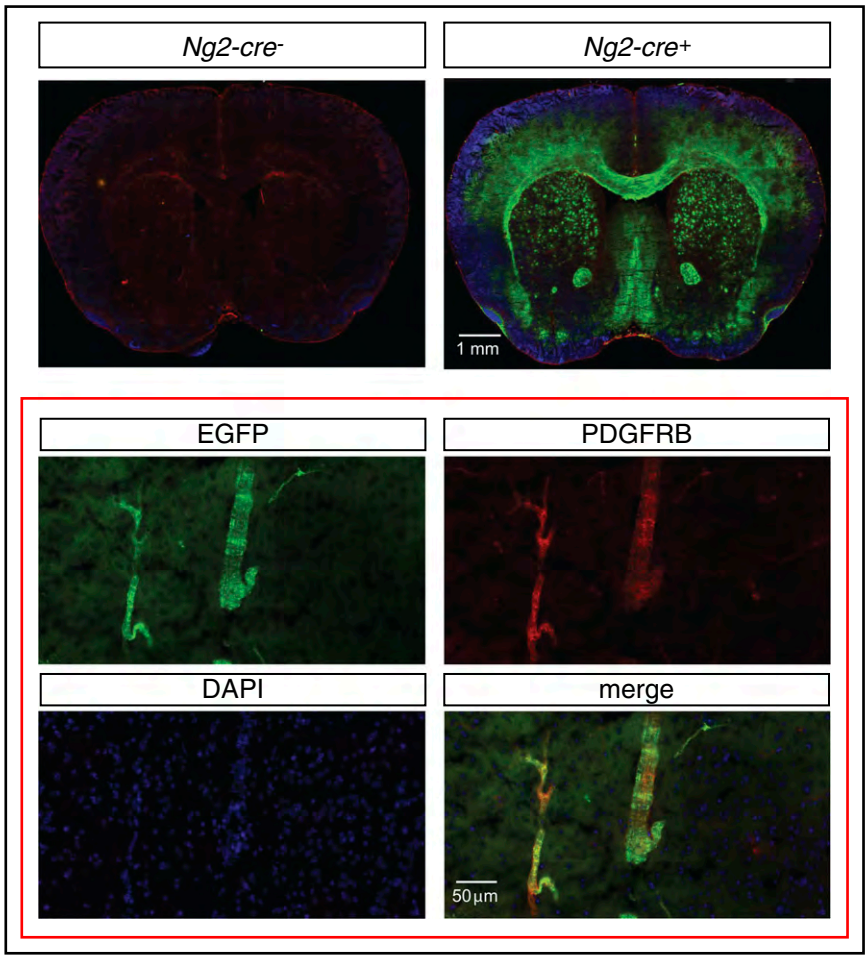

Figure 3. *Ng2-cre*–mediated recombination in cerebral vascular mural cells. (Top) Representative images of EGFP (green signal) and PDGFRB (red signal) immunofluorescence in brains from control (*Ng2-cre*$^{-}$) and NG2-*mT/mEGFP* mice that were deficient for *Phd2* (*Ng2-cre*$^{+}$). NG2-*mT/mEGFP-Phd2*$^{-/-}$ mice are phenotypically normal and did not develop polycythemia. (Bottom) Magnification shows coexpression of PDGFRB (red signal) and EGFP (green signal) in NG2 cells.

pericytes were capable of producing EPO in an experimental model of systemic hypoxia. For this, we placed wild-type mice in a normobaric hypoxia chamber for 24 hours and analyzed the contribution of pericytes to the brain's EPO response by multiplex RNA FISH. We exposed mice to 8% oxygen, which mimics an altitude of ~7500 m and is known to robustly stimulate EPO synthesis in the brain[12,13,46]; the contribution of pericytes to this response, however, has never been examined. After demonstrating increased *Epo* transcript levels (supplemental Figure 1A), we quantified the total number of *Epo*-expressing cells per brain subregion, as well as the number of cells that expressed pericyte marker *Pdgfrb*. Exposure of mice to 8% O_2 for 24 hours resulted in a widespread induction of *Epo* transcripts compared with normoxic controls, in which no signal was detected. Quantification of *Epo*$^{+}$ cells in relation to *Pdgfrb*-expressing cells revealed that ~80% of *Pdgfrb*$^{+}$ cells coexpressed *Epo* transcripts and that these cells represented 25% to 45% of the total number of *Epo*$^{+}$ cells (Figure 5). Interestingly, the number of *Epo*$^{+}$*Pdgfrb*$^{+}$ cells increased to ~70% under conditions of anemic hypoxia (Figure 5; relative *Epo* levels are shown in supplemental Figure 1B). *Epo*$^{+}$*Pdgfrb*$^{-}$ cells were identified as astrocytes and neurons (supplemental Figure 1C). Taken together, our data suggest that brain pericytes respond to hypoxia with the production of EPO, the degree of participation in this response being dependent on the type of the hypoxic stimulus.

PHD2 and PHD3 coregulate EPO production in NG2 cells

PHD enzymes function as the oxygen sensors that control HIF activity and thus play a central role in the oxygen-dependent induction of EPO.[27] In the kidney, EPO production can be stimulated by *Phd2* inactivation alone, which leads to a dramatic increase in renal *Epo* transcription,[4,35,47,48] whereas a combined inactivation of *Phds* is required to induce *Epo* in hepatocytes.[8,10] To investigate the role of individual PHDs in the regulation of EPO in brain pericytes, we generated mice with conditional deletions of *Phd1*, *Phd2*, and/or *Phd3* using *Ng2-cre*. We first inactivated *Phd2* in NG2-cells to examine whether *Phd2* deletion alone would mimic the phenotype of NG2-*Vhl*$^{-/-}$ mice. Conditional deletion of *Phd2* in NG2 cells (NG2-*Phd2*$^{-/-}$) did not result in polycythemia, abnormal plasma EPO, or increased brain *Epo* mRNA levels (Figure 6A), suggesting that *Phd2* deletion in NG2 cells was not sufficient to stabilize HIF-α and that the hypoxic regulation of *Epo* in brain pericytes differed from renal EPCs.

PHD1 and PHD3 have been shown to fine-tune HIF responses, especially under reoxygenation conditions, as PHD3 itself is hypoxia inducible.[49,50] To examine the role of PHD1 and PHD3 in pericytes, we generated compound conditional knockout mice in which PHDs were inactivated in combination. Whereas RBC parameters and plasma EPO in mice homozygously deficient for *Phd1* and *Phd2* and heterozygously deficient for *Phd3* (NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{+/-}$) or completely deficient for *Phd1* and *Phd3* but heterozygously deficient for *Phd2* (NG2-*Phd1*$^{-/-}$*Phd2*$^{+/-}$*Phd3*$^{-/-}$) were not different from littermate controls, the combined loss of either *Phd2* and *Phd3* (NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$) or *Phd1*, *Phd2*, and *Phd3* together (NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$) resulted in elevated plasma EPO concentration (two- and fourfold increase, respectively) and polycythemia, and thus mimicked the NG2-*Vhl*$^{-/-}$ phenotype (Figure 6A). In contrast to NG2-*Vhl*$^{-/-}$

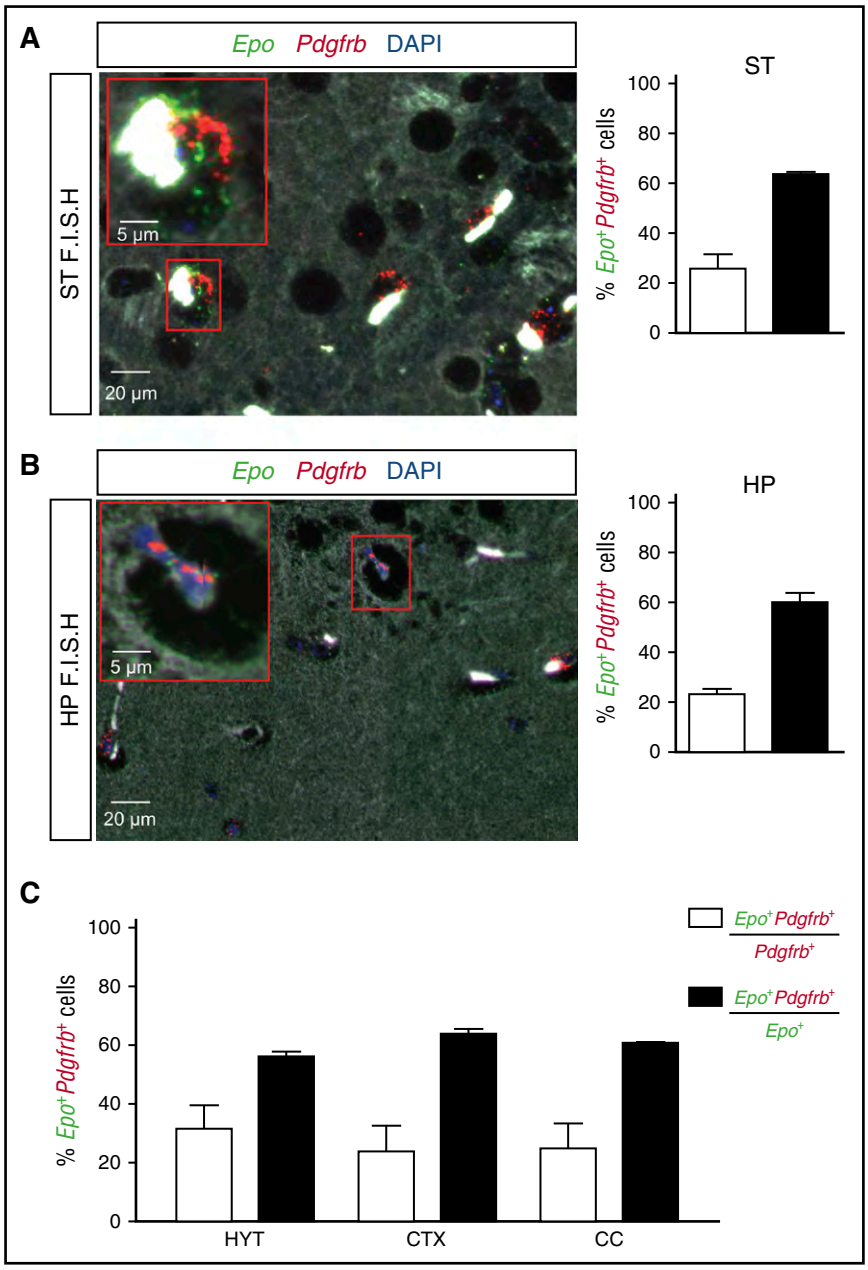

Figure 4. Brain pericytes express *Epo* mRNA. Shown are the results of multiplex RNA FISH studies for *Epo* and *Pdgfrb* transcripts using formalin-fixed, paraffin-embedded brain tissue sections from NG2-*Vhl*$^{-/-}$ mutant mice. (A) (Left) Representative image of a striatal section (ST) containing pericytes that coexpress *Epo* (green signal) and *Pdgfrb* transcripts (red signal). Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI; blue fluorescence). Bright white structures inside the vessels represent RBCs. (Right) Percentage of *Pdgfrb*$^{+}$ cells coexpressing *Epo* mRNA (white bar) or *Epo*$^{+}$ cells coexpressing *Pdgfrb* transcripts (black bar) (n = 3). (B) (Left) Representative image of a hippocampal section (HP) containing pericytes that coexpress *Epo* (green signal) and *Pdgfrb* transcripts (red signal). Nuclei are stained with DAPI (blue signal). (Right) Percentages of *Pdgfrb*$^{+}$ cells that coexpress *Epo* transcripts (white bar) or *Epo*$^{+}$ cells that coexpress *Pdgfrb* transcripts (black bar) (n = 3). (C) Percentages of *Pdgfrb*$^{+}$ cells coexpressing *Epo* transcripts (white bar) or *Epo*$^{+}$ cells that coexpress *Pdgfrb* transcripts (black bar) in hypothalamus (HYT), cortex (CTX), and corpus callosum (CC) (n = 3 each).

mice, *Epo* mRNA expression levels in kidney and bone from NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mutants were not different from controls. However, *Epo* mRNA and EPO protein levels in brain were strongly increased by 111- and 44-fold (8.9 ± 1.1 vs 390.6 ± 56.9 pg/mg total protein), respectively (Figure 6B). These results suggested that *Ng2-cre*–targeted cells in brain were responsible for the elevated plasma EPO levels found in NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice. Taken together, our data provide evidence that brain-derived EPO contributes to the regulation of erythropoiesis under conditions of PHD inactivation.

We hypothesized that the degree of contribution of brain-derived EPO to plasma EPO was most likely dependent on tissue EPO levels and blood–brain barrier (BBB) permeability, as only 1% of circulating EPO has been shown to cross the BBB under baseline conditions.[51-53] We therefore examined whether inactivation of *Phds* in NG2 cells affected BBB permeability. We found a significant increase in BBB permeability in NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice (supplemental Figure 2). This finding raises the possibility that *Phd* inactivation in NG2 cells may have facilitated delivery of brain pericyte-derived EPO to the systemic circulation through effects on BBB permeability.

Dual fluorescence RNA FISH in NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ brain tissue demonstrated that the distribution and cellular location of *Epo* transcripts was similar to NG2-*Vhl*$^{-/-}$ mice (data not shown). Although mice with complete inactivation of either *Phd2* and *Phd3* (NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$) or *Phd1*, *Phd2*, and *Phd3* together (NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$) developed severe polycythemia and were characterized by increased plasma EPO concentrations, the degree of polycythemia and increase in plasma EPO were more pronounced in NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ triple mutants (Figure 6A). However, we did not find a statistically significant difference in either brain *Epo* mRNA or EPO protein levels between NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ and NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice (Figure 6B-C). This is in contrast to the results from the endogenous IgG extravasation study, which suggested that BBB permeability was relatively more increased in NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ triple mutants, thus possibly

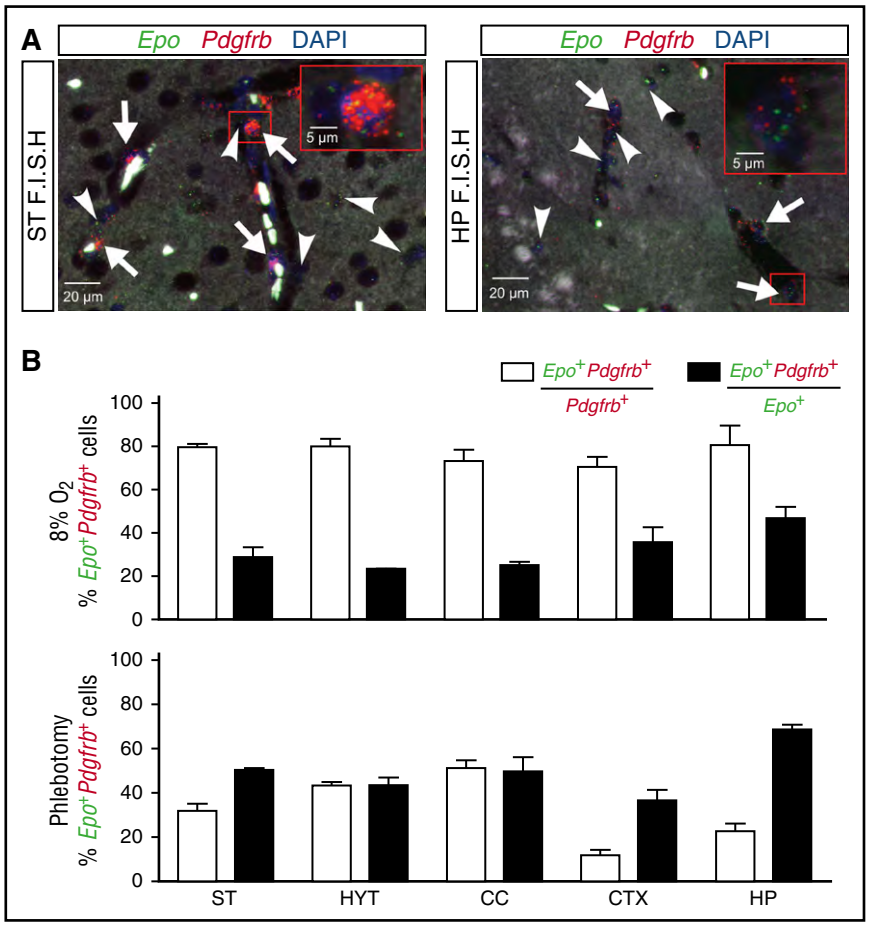

Figure 5. Normobaric hypoxia and anemia stimulate EPO synthesis in brain pericytes. Shown are the results of multiplex RNA FISH studies for *Epo* and *Pdgfrb* transcripts using formalin-fixed, paraffin-embedded brain tissue sections from wild-type mice exposed to normobaric hypoxia (8% O_2 for 24 hours) or after phlebotomy (average Hct: 20.7%). (A) (Left) Representative image of a striatal section (ST) containing pericytes (white arrows) that coexpress *Epo* (green signal) and *Pdgfrb* transcripts (red signal) and nonpericytic *Epo*$^+$ cells (white arrowhead). Nuclei are stained with DAPI (blue fluorescence). (Right) Representative image of a hippocampal section (HP) containing pericytes (white arrows) that coexpress *Epo* (green signal) and *Pdgfrb* transcripts (red signal) and *Pdgfrb*$^-$ *Epo*$^+$ cells (white arrowhead). Nuclei are stained with DAPI (blue signal). (B) (Upper) Percentages of *Pdgfrb*$^+$ cells that coexpress *Epo* (white bar) or *Epo*$^+$ cells that coexpress *Pdgfrb* transcripts (black bar) in striatum (ST), hypothalamus (HYT), corpus callosum (CC), cortex (CTX), and hippocampus (HP) from wild-type mice exposed to normobaric hypoxia (8% O_2 for 24 hours) (n = 3). (Lower) Percentages of *Pdgfrb*$^+$ cells that coexpress *Epo* (white bar) or *Epo*$^+$ cells that coexpress *Pdgfrb* transcripts (black bar) in striatum (ST), hypothalamus (HYT), corpus callosum (CC), cortex (CTX), and hippocampus (HP) from phlebotomized wild-type mice (n = 3).

facilitating the delivery of more EPO to the systemic circulation (supplemental Figure 2). Taken together, our results indicate that the hypoxic induction of EPO in brain pericytes is predominantly cocontrolled by PHD2 and PHD3. In this regard, EPO regulation in brain pericytes differs from the kidney, where *Phd2* inactivation alone is sufficient to induce EPO in a subset of perivascular cells.[4]

EPO production in NG2-Phd2$^{-/-}$Phd3$^{-/-}$ mice requires HIF-2

Although in vitro experiments demonstrated a role for HIF-1 in the regulation of EPO, genetic and immunohistochemical studies in humans and mice have now established that HIF-2 is the key transcription factor in the hypoxic induction of EPO in vivo.[9] Because of these findings, we focused our investigation on the role HIF-2 in *Phd*-deficient brain pericytes and generated mice with conditional inactivation of *Phd2*, *Phd3*, and *Hif2a*. Homozygous deletion of *Hif2a* in NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice corrected their polycythemic phenotype and suppressed brain *Epo* mRNA levels completely, establishing that under conditions of PHD inactivation, HIF-2 is required for EPO synthesis in brain pericytes (Figure 7).

Discussion

Here we used a genetic approach to investigate the PHD/HIF/VHL axis in NG2 cells. Our data establish that brain pericytes function as oxygen sensors and represent a major cellular source of EPO in the hypoxic brain, as they respond to systemic hypoxia with the induction of *Epo* transcription. We furthermore provide experimental evidence for a HIF-2–dependent contribution of brain pericytes to the regulation of erythropoiesis under conditions of combined inactivation of *Phd2* and *Phd3*.

To study the PHD/HIF/VHL axis in pericytes, we made use of a Cre-recombinase transgene under the control of the *Cspg4/Ng2* promoter. As NG2 is not only expressed in pericytes but also in polydendrocytes, which are cellular precursors of oligodendrocytes and protoplasmic astrocytes,[43] both pericytes and NG2-derived glia were subjected to Cre-mediated gene targeting in our model.[15,16] In the brain, pericytes can be easily differentiated from astrocytes and oligodendrocytes by their close association with vessel wall and capillaries, their morphologic features, and their expression of certain molecular markers such as PDGFRB.[16-18,45] We used these criteria to determine that the majority of EPO-producing cells in the brain of polycythemic *Ng2-cre*–based knockout mice were pericytes. As expected, we also found *Epo*-expressing cells that were *Pdgfrb*$^-$, which we identified as astrocytes and neurons, cell types that are known to synthesize EPO under conditions of hypoxia or *Vhl* inactivation.[12,13,54]

Pericytes play a key role in brain tissue homeostasis, as they are located at the interface of parenchyma and systemic circulation, where they control the formation, stability, and function of the BBB.[55,56] Pericytes regulate microvessel maturation and cerebral blood flow and are thus an integral part of the neurovascular unit.[16,45] Under conditions of systemic hypoxia, the majority of *Pdgfrb*$^+$ cells in wild-type mice expressed *Epo* and, depending on the hypoxic stimulus, accounted for ~30% to 70% of all *Epo*-expressing cells in the brain, suggesting that

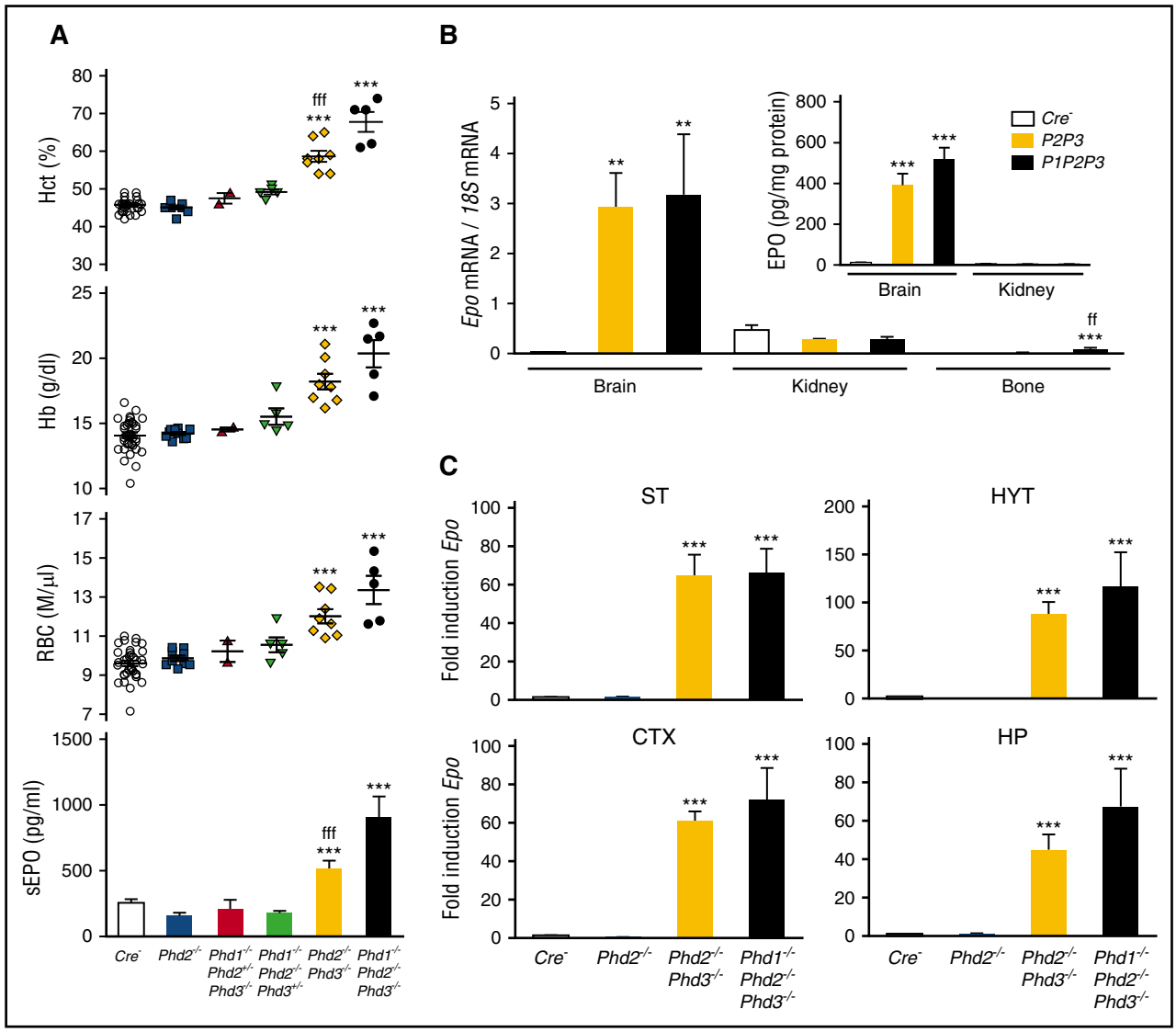

Figure 6. PHD2 and PHD3 control *Epo* transcription in NG2 cells. (A) Shown are Hct, RBC, and Hb values for individual Cre$^-$ control (n = 38), NG2-*Phd2*$^{-/-}$ (n = 9), NG2-*Phd1*$^{-/-}$ *Phd2*$^{+/-}$*Phd3*$^{-/-}$ (n = 2), NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{+/-}$ (n = 5), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (n = 8), and NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice (n = 5). Bar graphs show serum EPO levels (sEPO) for control (Cre$^-$, n = 18), NG2-*Phd2*$^{-/-}$ (n = 8), NG2-*Phd1*$^{-/-}$*Phd2*$^{+/-}$*Phd3*$^{-/-}$ (n = 2), NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{+/-}$ (n = 4), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (n = 5), and NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice (n = 4). Data are represented as mean ± SEM; 1-way ANOVA followed by Tukey's post hoc analysis; ***$P < .001$ compared with control group and $^{fff}P < .001$ when the NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ group was compared with the NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ group. (B) Relative *Epo* mRNA levels in brain, kidney, and bone from control (Cre$^-$), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$, and NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice (n = 3-7). (Inset) Brain and kidney EPO protein levels in Cre$^-$ control and NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (*P2P3*) and NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (*P1P2P3*) mice expressed as pg/mg total tissue protein (n = 3-6). Data are represented as mean ± SEM; 1-way ANOVA followed by Tukey's post hoc analysis; **$P < .01$, ***$P < .001$ compared with control group; $^{ff}P < .01$ for NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice compared with NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice. (C) Striatal (ST), hypothalamic (HYT), cortical (CTX), and hippocampal (HP) *Epo* mRNA levels in Cre$^-$ control (n = 9), NG2-*Phd2*$^{-/-}$ (n = 5), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (n = 4), and NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (n = 3). Data are represented as mean ± SEM; 1-way ANOVA followed by Tukey's post hoc analysis; ***$P < .001$ compared with Cre$^-$ control group.

pericytes function as oxygen sensors and contribute to at least one third of the brain's EPO response to hypoxia.

The implications of our findings for human biology implications are broad. The role of EPO in the biology of the neurovascular unit is unclear, and our findings will very likely stimulate further investigations in this area. Furthermore, phase 2 and 3 anemia clinical trials with HIF-stabilizing PHD inhibitors are currently under way.[2] Whether some of these compounds cross the BBB and induce *Epo* in brain pericytes is not known but warrants investigation.

Although the BBB is quite impermeable to EPO[51-53] and the contribution of brain-derived EPO to circulating EPO has been contested,[57] our data provide indirect evidence that brain pericyte-derived EPO may be able to reach the bloodstream. Although specific assays that permit plasma concentration measurements of tissue-specific EPO isoforms, eg, mass spectrometry–based assays, are currently not available, the analysis of polycythemic *NG2-Phd2*$^{-/-}$ *Phd3*$^{-/-}$ mice provides indirect evidence for a contribution of brain-derived EPO to the plasma EPO pool. In this mouse strain, which is characterized by an approximate twofold increase in plasma EPO levels, only 2 tissue sources of EPO were identified: brain and kidney. Because kidney EPO output did not change compared with control, our data support the notion that the increase in plasma EPO is brain derived, as brain was the only extrarenal tissue in which increased EPO was detected.

Although EPO is predicted to enter the systemic circulation directly in areas that lack a normal BBB, such as in the circumventricular organs or the choroid plexus,[58] the mechanism by which EPO crosses the BBB in other areas is not understood. However, under hypoxic conditions,

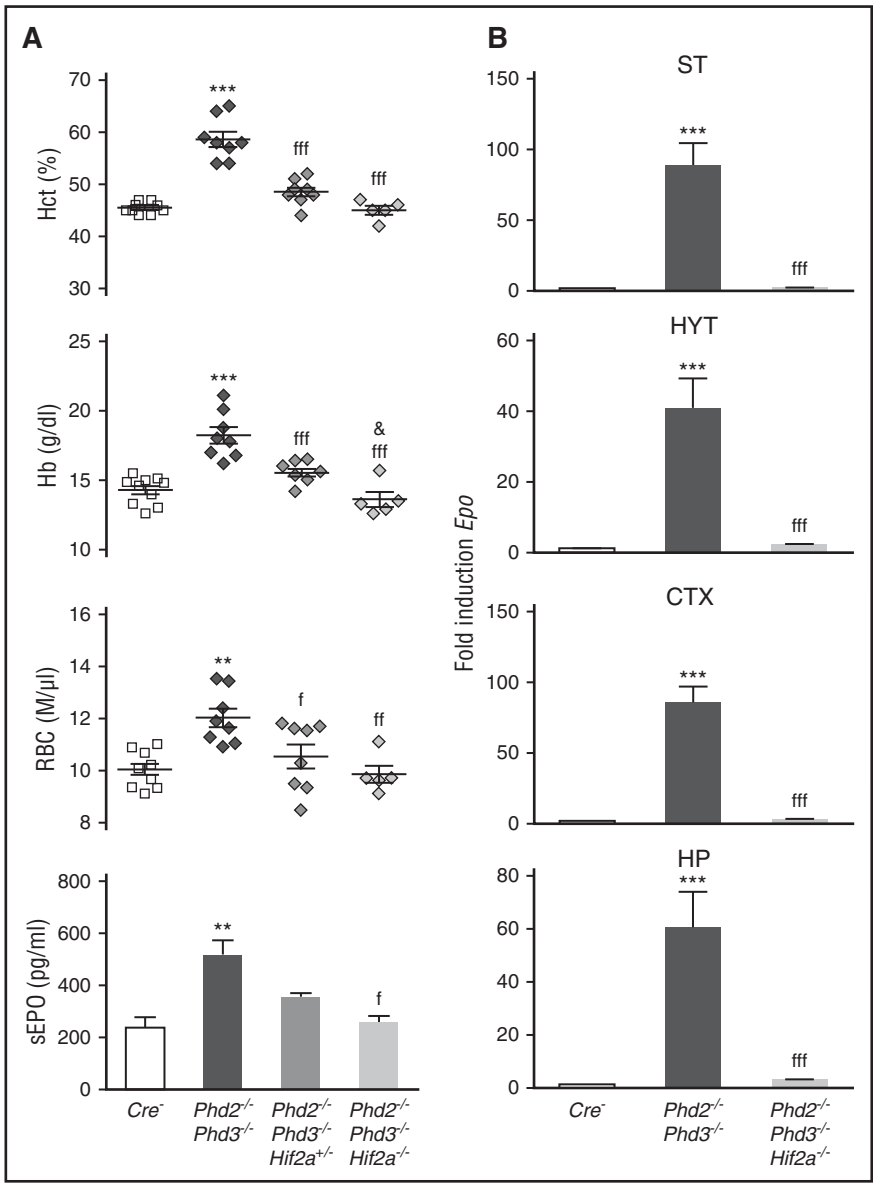

Figure 7. HIF-2 controls *Epo* transcription in NG2 cells. (A) Shown are individual Hct, RBC, and Hb values for Cre$^-$ control (n = 10), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (n = 8), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$*Hif2a*$^{+/-}$ (n = 8), and NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$*Hif2a*$^{-/-}$ mice (n = 5). Bar graphs show serum EPO levels (sEPO) for Cre$^-$ control (n = 4), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (n = 6), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$*Hif2a*$^{+/-}$ (n = 3), and NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$*Hif2a*$^{-/-}$ mice (n = 3). Data are represented as mean ± SEM; 1-way ANOVA followed by Tukey's post hoc analysis; $^{**}P < .01$ and $^{***}P < .001$ compared with Cre$^-$ control group; $^{f}P < .05$, $^{ff}P < .01$, and $^{fff}P < .001$ compared with NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ group; $^{\&}P < .05$, NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$*Hif2a*$^{+/-}$ was compared with NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$*Hif2a*$^{-/-}$ group. (B) Striatal (ST), hypothalamic (HYT), cortical (CTX), and hippocampal (HP) *Epo* mRNA levels in Cre$^-$ (n = 7), NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ (n = 4), and NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$*Hif2a*$^{-/-}$ mice (n = 4). Data are represented as mean ± SEM; 1-way ANOVA followed by Tukey's post hoc analysis; $^{***}P < .001$ compared with Cre$^-$ control group and $^{fff}P < .001$ compared with the NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ group.

the endothelial barrier becomes leaky,[59] which is predicted to facilitate the delivery of pericyte-derived EPO into the blood stream. We believe that the severity and duration of hypoxia are important variables that affect BBB permeability[60] and thus likely modulate to what degree brain-derived EPO enters the systemic circulation.

In adults, the main source of EPO are renal peritubular interstitial fibroblast-like cells, which represent a heterogeneous and relatively ill-defined population of cells that includes pericytes and perivascular fibroblasts.[2] Recent studies have indicated that the majority of interstitial fibroblast-like cells in the renal cortex and outer medulla have the capacity to synthesize EPO in a HIF-2–dependent fashion,[4] whereas morphologic and molecular analysis indicates that a large fraction of renal EPCs are pericytes.[3,4,61] Interestingly, lineage tracing studies have suggested that pericytes in brain and kidney are neural crest derived[5,18,19] and that HIF-2α expression is associated with a neural crest–like phenotype.[62,63] Thus, the ability to produce EPO may be a functional feature of pericytes of neural crest origin.

Although NG2 is marker of pericytes, only a small subpopulation of *Pdgfrb*-expressing renal pericytes is targeted by *Ng2-cre* (supplemental Figure 3). Inactivation of either *Vhl* or *Phd2/3* in these cells resulted in constitutive HIF-2 activation and led to inappropriate EPO production, as renal synthesis EPO is normally suppressed in the presence of polycythemia. In contrast to NG2-*Vhl*$^{-/-}$ mice, renal *Epo* levels in NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ and NG2-*Phd1*$^{-/-}$*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice were lower, although statistically not significant, whereas in the bone *Epo* was induced in the *Vhl* and triple *Phd* knockout but not in NG2-*Phd2*$^{-/-}$*Phd3*$^{-/-}$ mice. Phenotypic differences between *Vhl* and the 2 *Phd* knockout models are expected as cell type–specific regulation of HIF activity and EPO synthesis has been associated with differences in PHD expression levels and catalytic activity,[4] whereas *Vhl* inactivation leads to a complete block of HIF ubiquitylation, representing the least physiologic model of HIF activation.

We were able to demonstrate that PHD2 and PHD3 coregulate EPO production in brain pericytes, as the combined inactivation of *Phd2* and *Phd3* was required for *Epo* induction. This is in contrast to the kidney, where *Phd2* inactivation alone is sufficient for *Epo* induction in a subset of cells.[4,35,47,48] PHD2 is the main HIF prolyl-4-hydroxylase that controls HIF activity under normoxia,[27] and loss-of-function mutations are associated with familial erythrocytosis in humans,[64,65] whereas gain-of-function mutations protect from high altitude–induced polycythemia or

other diseases.[1,66,67] However, only a subset of renal interstitial cells responds to *Phd2* inactivation alone.[4] It is not surprising that cells with EPO-producing capacity behave differently with regard to HIF pathway regulation and thus EPO production, as the combined deletion of ≥2 PHDs is required to increase EPO production in hepatocytes, whereas the inactivation of all 3 PHDs is needed for a very strong and sustained *Epo* induction in the liver.[8,10] These tissue- and cell type–dependent differences are most likely a reflection of differences in PHD protein expression levels and catalytic activity.

In summary, our studies provide novel insights into the regulation of hypoxia responses in the brain microvasculature, as we provide experimental evidence for a role of brain pericytes as oxygen sensors. Our findings provide a basis for further research into the role of oxygen metabolism in brain microvascular function and pathogenesis.

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Authorship

Contribution: A.A.U. and V.H.H. conceived and designed the research studies, analyzed and interpreted data, wrote the manuscript, and made the figures; A.A.U., A.A., J.N., and O.D. performed experiments and acquired and analyzed data; and K.W.G. contributed reagents and interpreted data.

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ORCID profiles: A.A.U., 0000-0001-7945-5515; A.A., 0000-0001-6406-2233; V.H.H., 0000-0002-7051-8994.

Correspondence: Volker H. Haase, Division of Nephrology and Hypertension, Vanderbilt University Medical Center, C-3119A MCN, 1161 21st Ave S, Nashville, TN 37232-2372; e-mail: volker.haase@vanderbilt.edu.

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Prolyl-4-hydroxylase 2 and 3 coregulate murine erythropoietin in brain pericytes

Andres A. Urrutia, Aqeela Afzal, Jacob Nelson, Olena Davidoff, Kenneth W. Gross and Volker H. Haase

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