ORIGINAL RESEARCH

Treatment With Tetrahydrobiopterin Improves White Matter Maturation in a Mouse Model for Prenatal Hypoxia in Congenital Heart Disease

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***Background*—**Reduced oxygen delivery in congenital heart disease causes delayed brain maturation and white matter abnormalities in utero. No treatment currently exists. Tetrahydrobiopterin (BH4) is a cofactor for neuronal nitric oxide synthase. BH4 availability is reduced upon NOS activation, such as during hypoxic conditions, and leads to toxin production. We hypothesize that BH4 levels are depleted in the hypoxic brain and that BH4 replacement therapy mitigates the toxic effects of hypoxia on white matter.

***Methods and Results*—**Transgenic mice were used to visualize oligodendrocytes. Hypoxia was introduced during a period of white matter development equivalent to the human third trimester. BH4 was administered during hypoxia. BH4 levels were depleted in the hypoxic brain by direct quantification (n=7–12). The proliferation (n=3–6), apoptosis (n=3–6), and developmental stage (n=5–8) of oligodendrocytes were determined immunohistologically. Total oligodendrocytes increased after hypoxia, consistent with hypoxia-induced proliferation seen previously; however, mature oligodendrocytes were less prevalent in hypoxia, and there was accumulation of immature oligodendrocytes. BH4 treatment improved the mature oligodendrocyte number such that it did not differ from normoxia, and accumulation of immature oligodendrocytes was not observed. These results persisted beyond the initial period of hypoxia (n=3–4). Apoptosis increased with hypoxia but decreased with BH4 treatment to normoxic levels. White matter myelin levels decreased following hypoxia by western blot. BH4 treatment normalized myelination (n=6–10). Hypoxia worsened sensory-motor coordination on balance beam tasks, and BH4 therapy normalized performance (n=5–9).

***Conclusions*—**Suboptimal BH4 levels influence hypoxic white matter abnormalities. Repurposing BH4 for use during fetal brain development may limit white matter dysmaturation in congenital heart disease. **(*J Am Heart Assoc*. 2019;8:e012711. DOI: 10.1161/JAHA.119.012711.)**

Key Words: congenital heart disease • hypoxia • neuroprotection • tetrahydrobiopterin

Congenital heart disease (CHD) affects ≈1% of births in the United States, amounting to 40 000 babies born with CHD each year.[1,2] Children with CHD are at a markedly increased risk for neurodevelopmental pathologies and behavioral problems.[3–6] Up to one half of infants with complex CHD have evidence of white matter abnormalities.[7–11] The most common neurologic deficits seen in infants after CHD repair are motor deficits,[4,12] suggestive of diffuse white matter pathology.[13] Additionally, behavioral problems include attention deficit hyperactivity disorder, executive dysfunction, impairment of working memory, and verbal dysfunction,[3,14,15] which are also associated with white matter abnormalities.[16–22]

Fetal blood flow normally directs the most oxygenated blood preferentially to the developing brain,[23] but the fetus with complex CHD (eg, single-ventricle lesions or dextro-transposition of the great arteries) is subject to alterations in blood flow and oxygen delivery to the brain while in utero.[23–26] The resulting desaturated or reduced blood flow to the brain causes impaired macroscopic brain growth, periventricular leukomalacia, parenchymal metabolic derangements, and delayed microscopic brain development.[23–25,27]

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Clinical Perspective

What Is New?

- To our knowledge, this is the first study to demonstrate that treatment with tetrahydrobiopterin (BH4) mitigates the deleterious effects of chronic hypoxia on developing white matter.
- Treatment with BH4 normalized the number of mature and immature oligodendrocytes, amount of myelin production, prevalence of apoptotic cells, and sensory-motor coordination such that BH4-treated animals did not differ from normoxic controls.

What Are the Clinical Implications?

- Repurposing BH4 for maternal administration during fetal brain development has the potential to reduce white matter dysmaturation seen in cyanotic congenital heart disease.
- Because BH4 is already approved by the Food and Drug Administration and shown to be safe during pregnancy, there is significant translational potential for BH4 to become a new neuroprotective therapy for fetuses with cyanotic congenital heart disease.

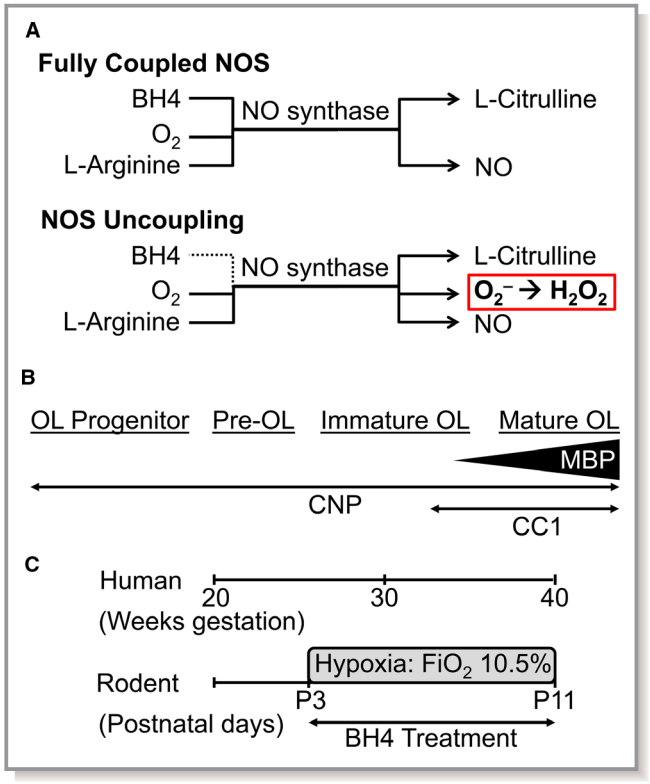

Figure 1. **A**, Nitric oxide synthase (NOS) catalysis under fully coupled NOS and NOS uncoupling. **B**, Antibody markers used to immunostain distinct developmental stages of oligodendrocyte lineage cells. **C**, Study design and equivalent time period for white matter development between the mouse and human. BH4 indicates tetrahydrobiopterin; FiO_2, fractional inspired oxygen concentration; MBP, myelin basic protein; NO, nitric oxide; OL, oligodendrocyte.

Furthermore, preoperative brain immaturity places the neonate at increased risk for neurologic injury during surgical repair of their CHD.[8,10] Thus, to improve the neurologic outcomes of children with CHD, we must turn our attention to the neurodevelopment of the fetus with CHD and protection against onset of hypoxic brain abnormalities during fetal life.[28,29]

Our research focuses on an important molecular cascade involved with hypoxic-ischemic injury. The first event in brain hypoxia is failure of the transporter that removes glutamate from the synaptic gap.[30,31] This causes an accumulation of glutamate and excessive N-methyl-D-aspartate (NMDA) receptor activation, which in turn causes calcium flooding, leading to increased activation of neuronal nitric oxide synthase (nNOS).[32,33] Tetrahydrobiopterin (BH4) is an important cofactor for nNOS, and in its absence, production of toxic peroxynitrite, termed *NOS uncoupling,* is favored[34] (Figure 1A). Peroxynitrite causes primary DNA damage and increases the production of reactive oxygen species, activating the apoptosis-necrosis continuum.[32,33] Increased nNOS activation has also been linked to white matter demyelination.[35]

Injury mediated by glutamate receptors is especially important in the developing brain because these receptors are functionally upregulated during the perinatal period, when they play a role in neuronal plasticity.[36] The most prominent cell in white matter is the oligodendrocyte, which is responsible for myelination of white matter in the brain and spinal cord. Glutamate receptor–mediated injury has been especially well observed in oligodendrocytes.[37,38]

In short, hypoxia leads to excessive stimulation of NMDA receptors and activation of nNOS, which produces peroxynitrite in the absence of BH4. These are crucial events in the development of hypoxic-ischemic brain pathology.[39,40] In addressing this glutamate receptor–mediated mechanism of hypoxic injury, it is not feasible to block the action of either NMDA receptors or nNOS, as they have important physiologic roles in the developing brain.[41,42] However, preventing the depletion of BH4, and thus decreasing the production of molecular toxins, has the potential to limit fetal brain abnormalities.

We performed this study in an animal model of chronic hypoxia to simulate hypoxic conditions in the prenatal brain, as would be found in cyanotic CHD. We hypothesize that BH4 levels are depleted in the hypoxic brain and that this depletion plays a critical role in developing hypoxic white matter derangements during brain development of the fetus with

CHD. Further, we hypothesize that treating with BH4 will mitigate the toxic effects of hypoxia on the developing white matter.

Materials and Methods

The data that support the findings of this study and further details regarding analytic methods and study materials can be made available to other researchers upon reasonable request to the corresponding author.

Animals

CNP-EGFP mouse pups[43] were used to allow for direct visualization of oligodendrocytes. CNP (2′,3-cyclic nucleotide 3′phosphodiesterase) is expressed by oligodendrocytes at all stages of cell maturation (Figure 1B). In transgenic mice, human EGFP (enhanced green fluorescent protein) is overexpressed in the oligodendrocyte lineage under the CNP promoter. The CNP-EGFP strain was generated as described previously and backcrossed to a C57BL/6 genetic background >9 generations.[44] Only mice that expressed green fluorescent protein during screening on postnatal day 2 with ultraviolet goggles were used. We performed all experiments in compliance with the *NIH Guide for the Care and Use of Laboratory Animals*. The study was approved by the Animal Care and Use Committee of the Children's National Health System.

Hypoxia Experiment and BH4 Treatment

The maturation state of white matter from postnatal day 3 (P3) to P11 in the mouse is the equivalent time period to the third trimester of pregnancy in humans (Figure 1C).[45,46] The hypoxic rearing therefore began on (P3) and continued until P11 in a hypoxic chamber system (BioSpherix, Redfield, NY) to reproduce the hypoxic state of the CHD fetus (Figure 1C).[46–48] A recent clinical magnetic resonance imaging study has identified that during the third trimester there is a progressive and significant decline in gestational age–adjusted white matter volume in CHD fetuses relative to normal controls.[49] During the experiment, the oxygen concentration was maintained, monitored, and recorded continuously with sensors placed inside the chamber to achieve a level of 10.5 ± 0.5% (Pro:Ox Model 360, BioSpherix) (Figure 1C).[45] Nitrogen gas was used to displace the oxygen and maintain hypoxic conditions (hypoxia). The temperature and humidity were monitored, and each of these parameters inside the chamber was similar to the normoxic condition (normoxia). Age-matched mice reared in normal oxygen levels were used to simulate normoxia. BH4 (Kuvan; BioMarin Inc, San Rafael, CA) at 50 mg/kg/day was administered orally over this same time period in our treatment group (Figure 1C), thus modeling initiation of therapy at the beginning of the third trimester —a common time for CHD diagnosis (hypoxia with BH4). Mice were randomly allocated to the 3 treatment groups, with no difference in sex distribution among the groups.

Liquid Chromatography Coupled to Electrospray Tandem Mass Spectrometry

Fresh frozen whole brain and whole heart samples at P11 (n=7–12) were thawed in ice at room temperature, homogenized in methanol and buffer containing 0.2 mol/L trichloroacetic acid, 1 mmol/L ethylenediaminetetraacetic acid, 6.5 mmol/L dithierythritol, and 50 mmol/L ascorbic acid, then vortexed and kept on ice for 10 minutes. After centrifugation at 1741*g* for 20 minutes at 4°C, the supernatants were resolved on an Acquity BEH C18 1.7 μm, 2.1×100 mm column online with a triple quadrupole mass spectrometer (Xevo-TQ-S; Waters Corporation, Milford, MA) operating in the multiple reaction monitoring mode. The instrument parameters were optimized to gain maximum specificity and sensitivity of ionization for the parent (m/z 242.213; BH4) and daughter ions (m/z 166.08). Signal intensities from all multiple reaction monitoring Q1/Q3 ion pairs for the analyte were ranked to ensure selection of the most intense precursor and fragment ion pair for multiple reaction monitoring–based quantitation. Multiple reaction monitoring data were processed using Target Lynx 4.1. The relative quantification values of analytes were determined by calculating the ratio of peak areas of transitions of samples normalized to the peak area of the internal standard.

Immunohistochemistry

At the conclusion of each experiment, mice were deeply anesthetized and perfused transcardially with 20 mL of 0.1 mol/L phosphate buffer saline, followed by 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde for 24 hours at 4°C, and cryoprotected for at least 48 hours in phosphate buffer saline containing 30% sucrose. All brains were stored at −80°C until further processing. For assessment of white matter oligodendrocytes, 20-μm sections were incubated for 1 hour at room temperature with blocking solution (10% normal goat serum, 1% bovine serum albumin, and 0.3% Tween 20 in phosphate buffered saline, pH 7.4) and then incubated at 4°C overnight with primary antibody and carrier solution (1% normal goat serum, 1% bovine serum albumin, and 0.3% Tween 20 in phosphate-buffered saline, pH 7.4). Sections were washed with phosphate-buffered saline and then

incubated for 1 hour at room temperature with secondary antibody and carrier solution. We identified mature oligodendrocytes using the CC1 antibody, which binds quaking protein 7, an RNA-binding protein highly upregulated in myelinating oligodendrocytes (Figure 1B), as we performed previously[50] (n=5–8 at P11; n=3–4 at P30). Antibodies to cleaved caspase-3 were used to identify apoptosis in each developmental stage of white matter oligodendrocytes (n=3–6). For cellular analysis, mouse white matter was subdivided into the following 3 regions: (1) corpus callosum; (2) cingulum; and (3) external capsule. To determine cell density, the antibody-positive cells were quantified using a stereological counting system (Stereo Investigator; MBF Bioscience, Williston, VT). The system provides systematic random sampling to obtain unbiased estimates of cell number.[51]

Western Blot

MBP (myelin basic protein) expression level by western blotting indicates degree of myelination (Figure 1B). For western blot analysis, tissues were precisely dissected from coronal sections among tested groups (n=6–10). Tissues were homogenized in lysis buffer with proteinase inhibitors. Protein extracts were boiled for 5 minutes before loading onto 4% to 20% gradient gels. Gels were electrotransferred to a 0.2-μm nitrocellulose membrane. Blots were blocked and then incubated with an antibody against MBP. Band intensity was measured using the ImageJ program (National Institutes of Health).

Inclined-Beam Testing

The inclined-beam test was used to assess sensory–motor integration and the overall function of the subcortical white matter.[44] Animals were kept on a regular 12-hour light/dark cycle. Food and water were made available ad libitum. Mice were acclimated to the behavioral testing room for 1 hour before commencement of testing. An 80-cm-long and 1-cm-wide wooden beam was placed at a 30-degree angle. A dark box with bedding was at the end of the incline and served as a target for the mouse to reach. A blinded experimenter assessed performance by documenting the number of hind-leg foot slips and the time to traverse the beam. The beam was cleaned with 30% ethanol (v/v) between each mouse. Males and females were used for this experiment (n=5–9).

Statistical Analysis

The Kolmogorov–Smirnov goodness-of-fit test assessed whether the continuous variables follow a normal (Gaussian-shaped) distribution. A 2-tailed, unpaired Student *t* test was performed for single comparisons. For multiple comparisons, a 1-way or 2-way ANOVA was applied with the F-test to determine group difference and Bonferroni post hoc comparisons to protect against type 1 errors (false-positive results). Statistical analysis was performed using Prism software (GraphPad Software, Inc, La Jolla, CA). All data are expressed as mean±SEM. All *P* values were 2-tailed using an α-level of 0.05 as the criterion for statistical significance.

Results

Chronic Hypoxia Depletes BH4 Levels in the Developing Brain

In previous studies, BH4 was indirectly quantified by high-performance liquid chromatography.[52,53] However, recent studies have shown direct measurement with liquid chromatography–tandem mass spectrometry to be a more accurate method.[54] To test our hypothesis that BH4 levels are depleted in the hypoxic brain, we quantified BH4 levels in the brain and heart using the direct tissue detection method (Figure 2A). Our direct quantification found that brain BH4 levels were significantly lower in hypoxia compared with normoxia (−38.4%; *P*=0.02; Figure 2B). We also tested heart tissue levels of BH4 to see if there were any potential targets of therapy there as well; however, there was no difference in heart BH4 levels between hypoxia and normoxia (Figure 2C). These results indicate BH4 to be a relevant substance in hypoxic events in the developing brain, thus establishing a potential phramacologic target for improving outcomes in chronic hypoxia through replacement therapy.

BH4 Treatment Does Not Interfere With Expansion of Oligodendrocyte Pool Caused by Hypoxia

Because hypoxia caused by complex CHD affects white matter, we examined the effects of hypoxia and BH4 treatment on the development of oligodendrocytes in our animal model. When we analyzed the number of CNP^+ entire oligodendrocyte lineages (Figure 1B) in 3 white matter tracts at P11, the number significantly varied according to treatment group but not by white matter region (Figure 3A through 3D). Consistent with hypoxia-induced oligodendrocyte proliferation seen previously,[47] our results demonstrated that CNP^+ oligodendrocyte number increased with hypoxia as compared with normoxia (Figure 3A, 3B, and 3D). Treatment with BH4 did not limit this hypoxia-induced proliferation; CNP^+ cell number was also increased for hypoxia with BH4 as compared with normoxia (Figure 3A, 3C, and 3D) such that there was no difference between hypoxia and hypoxia with BH4 (Figure 3B

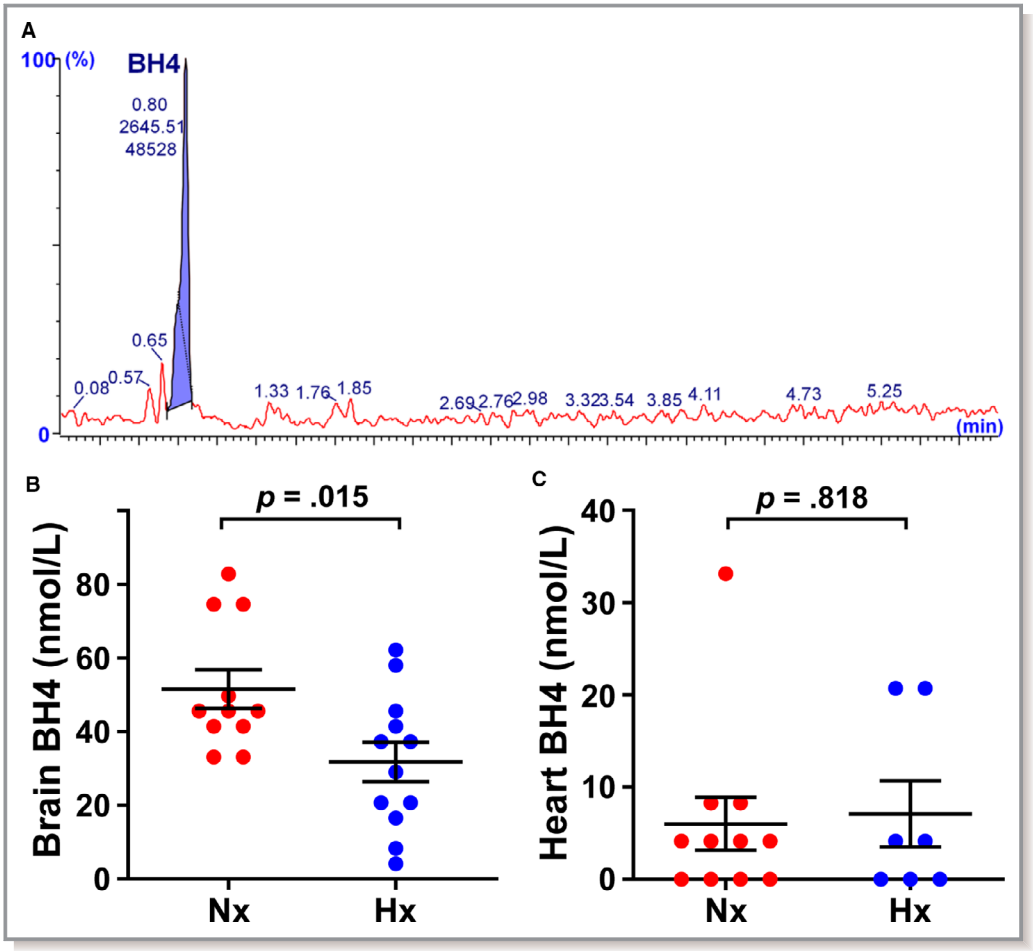

Figure 2. Chronic hypoxia depletes BH4 levels in the developing brain. **A**, Direct quantification of BH4 using liquid chromatography coupled to electrospray tandem mass spectrometry. **B** and **C**, BH4 levels are significantly lower following hypoxia in the brain (**B**); however, there is no difference in heart BH4 levels between normoxia and hypoxia (**C**). Data are shown as mean±SEM (n=7–12 each). BH4 indicates tetrahydrobiopterin; Hx, hypoxia; Nx, normoxia.

through 3D). This indicates that BH4 treatment does not interfere with the aforementioned hypoxia-induced expansion of the oligodendrocyte pool in developing white matter.

Increases in Apoptosis During Hypoxia Are Mitigated By BH4, but BH4 Treatment Does Not Change Apoptosis Within Oligodendrocyte Lineages

Reduction of brain BH4 levels from nNOS activation during hypoxia causes increased production of toxic peroxynitrite, which triggers cell apoptosis. Next, we assessed the effects of hypoxia and BH4 treatment on caspase-3 activation in the developing white matter. Number of cleaved (active) caspase-3$^+$ cells varied by treatment group but not by white matter region (Figure 4A through 4D). Consistent with previous findings in this model,[44,47] caspase-3$^+$ cell number increased with hypoxia as compared with normoxia (Figure 4A, 4B, and 4D). Treatment with BH4 significantly decreased apoptosis, as hypoxia with BH4 had fewer caspase-3$^+$ cells than hypoxia (Figure 4B through 4D). Remarkably, there was no difference in caspase-3$^+$ cell number between normoxia and hypoxia with BH4 (p=0.841), indicating that BH4 treatment normalized caspase activation during white matter development. When percentage of oligodendrocytes expressing cleaved caspase-3 (ie, apoptotic oligodendrocytes) were assessed, there were significant differences among the 3 treatment groups (Figure 4E). Consistent with aforementioned findings, chronic hypoxia caused significant increases in oligodendrocyte apoptosis as compared with normoxia (Figure 4E). Treatment with BH4 significantly decreased apoptotic cell population in the white matter (Figure 4D); however, we found no differences in apoptotic oligodendrocyte numbers between hypoxia and hypoxia with BH4 at a dose of 50 mg/kg per day (Figure 4E).

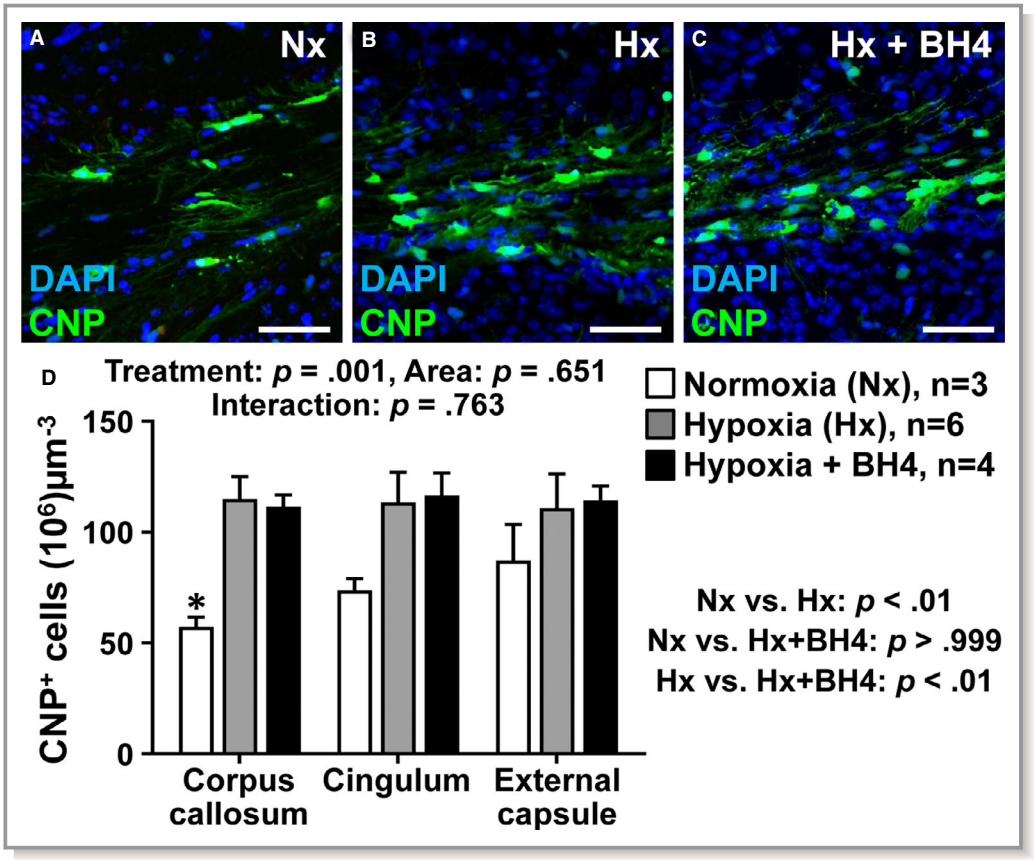

Figure 3. BH4 treatment does not interfere with expansion of oligodendrocyte pool due to hypoxia. **A** through **C**, CNP^+ oligodendrocyte lineages in the corpus callosum among 3 groups including normoxia, hypoxia, and hypoxia with BH4 treatment. **D**, CNP^+ oligodendrocyte number increased following hypoxia as compared with normoxia; treatment with BH4 does not affect hypoxia-induced oligodendrocyte proliferation. *P<0.01 vs hypoxia and P<0.01 vs hypoxia+BH4 by ANOVA with Bonferroni comparisons. Bar=50 µm. Data are shown as mean±SEM (n=3–6 each). BH4 indicates tetrahydrobiopterin; CNP, 2′,3-cyclic nucleotide 3′phosphodiesterase; DAPI, 4′,6-diamidino-2-phenylindole.

Treatment With BH4 Under Hypoxic Conditions Improves Hypoxia-Induced Arrested Oligodendrocyte Maturation

Next, we examined the maturation of oligodendrocytes in the same white matter tracts. Mature oligodendrocytes uniquely display CC1, which we targeted as a cell marker for this investigation (Figure 1B). Number of $CC1^+$ oligodendrocytes varied by treatment group but not by brain region (Figure 5A through 5D). Although CNP^+ entire oligodendrocyte lineages increased with hypoxia (Figure 3D), the number of $CC1^+$ mature oligodendrocytes significantly decreased with hypoxia as compared with normoxia (Figure 5A, 5B, and 5D). When we assessed CNP^+CC1^- immature oligodendrocyte numbers, expansion of the cell population was observed with hypoxia (Figure 5E), indicating arrested oligodendrocyte maturation attributable to chronic hypoxic conditions. These findings are consistent with preoligodendrocyte accumulation after perinatal ischemia-reperfusion injury in other rodent models.[55]

Remarkably, hypoxia-induced reduction of mature oligodendrocyte population was not seen under treatment with BH4 as hypoxia with BH4 showed more $CC1^+$ cells than did hypoxia (Figure 5B through 5D), and there was no difference between normoxia and hypoxia with BH4 in number of mature oligodendrocytes (Figure 5A, 5C, and 5D). Consistently, we observed no accumulation of CNP^+CC1^- immature oligodendrocyte numbers after BH4 treatment during hypoxia (Figure 5E), demonstrating that BH4 replacement therapy under hypoxic conditions improves hypoxia-induced arrested oligodendrocyte maturation.

Investigation of oligodendrocyte maturity at P30 demonstrated that the effects of hypoxia-induced arrested oligodendrocyte maturation persisted beyond the initial period of hypoxia. Hypoxia from P3 to P11 resulted in persistently decreased numbers of CNP^+CC1^+ mature oligodendrocytes at P30 when compared with both normoxia and hypoxia with BH4 (Figure 6A through 6D). Again, there was no

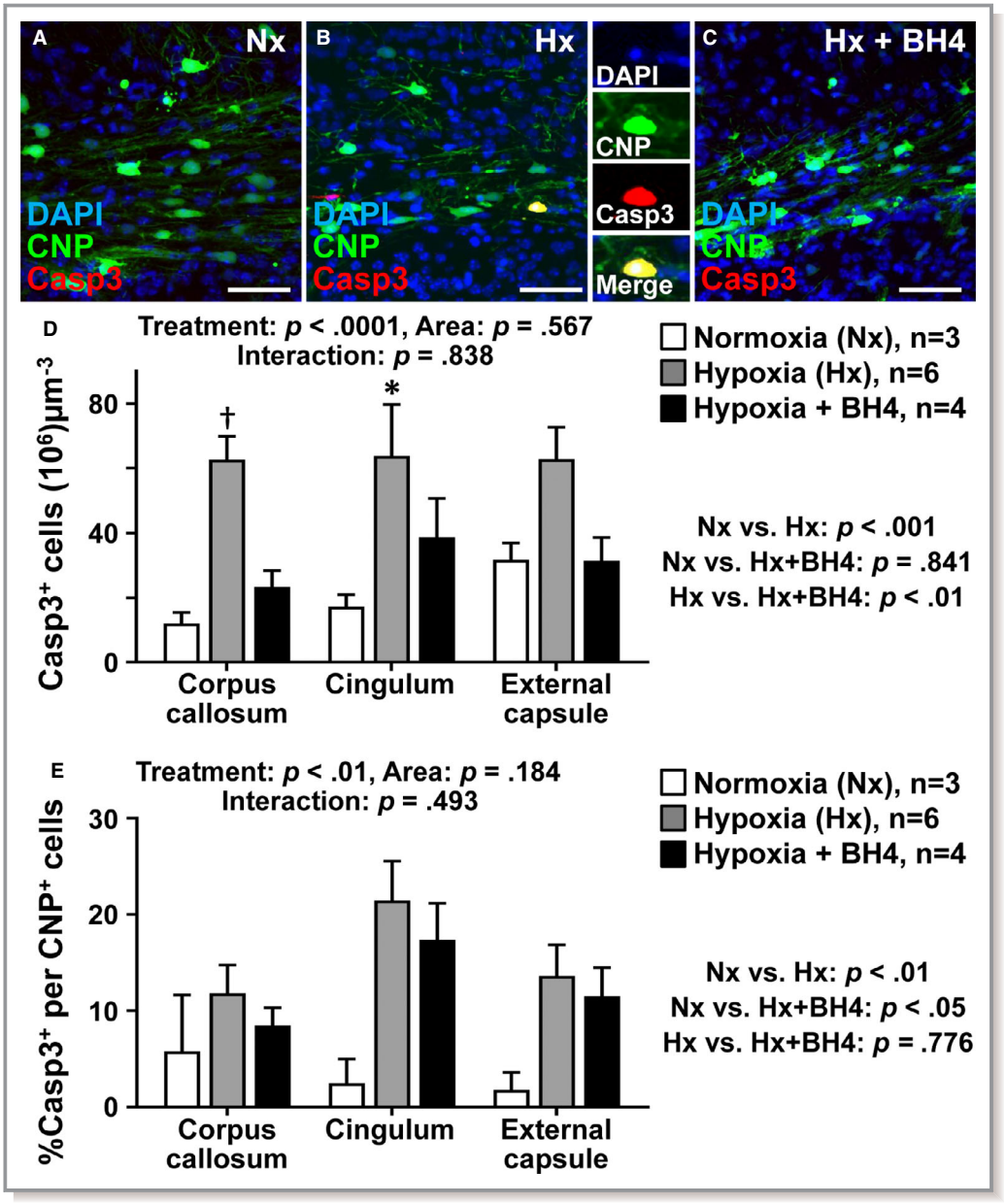

Figure 4. Increases in apoptosis during hypoxia are mitigated by BH4; however, BH4 treatment does not change apoptosis within oligodendrocyte lineages. **A** through **C**, CNP^+ caspase-3^+ apoptotic oligodendrocytes in the corpus callosum among 3 groups. **D**, Caspase-3^+ cell number increases following hypoxia. Treatment with BH4 reduces the number compared with hypoxia. There is no difference in caspase-3^+ cell number between normoxia and hypoxia with BH4. **E**, There is no difference in apoptotic oligodendrocyte numbers between hypoxia and hypoxia with BH4. *P<0.05 vs normoxia, $^{\dagger}P$<0.01 vs normoxia and P<0.05 vs hypoxia+BH4 by ANOVA with Bonferroni comparisons. Bar=50 µm. Data are shown as mean±SEM (n=3–6 each). BH4 indicates tetrahydrobiopterin; CNP, 2′,3-cyclic nucleotide 3′phosphodiesterase; DAPI, 4′,6-diamidino-2-phenylindole.

difference in CNP^+CC1^+ mature oligodendrocyte quantity between normoxia and hypoxia with BH4 (Figure 6A, 6C, and 6D), demonstrating that the benefits of BH4 treatment on hypoxia-induced arrested oligodendrocyte maturation persisted beyond the initial period of hypoxia.

Treatment With BH4 Eliminates Delayed Myelination Resulting From Chronic Hypoxia

We investigated the myelination performed by these oligodendrocytes by looking at expression of MBP.

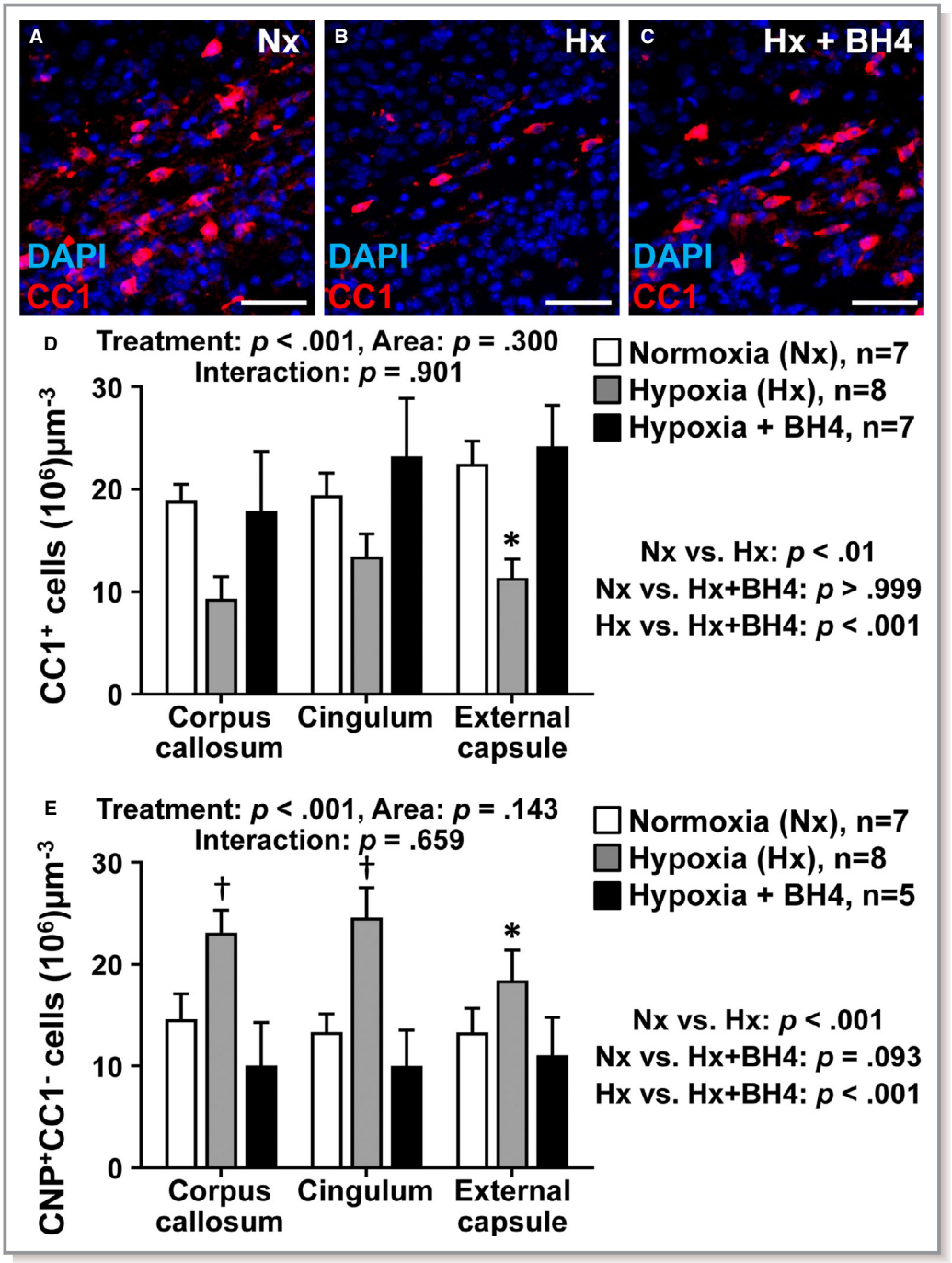

Figure 5. Treatment with BH4 under hypoxic conditions improves hypoxia-induced arrested oligodendrocyte maturation. **A** through **C**, $CC1^+$ mature oligodendrocytes in corpus callosum among 3 groups. **D**, Mature oligodendrocyte number decreases with hypoxia; on the other hand, the reduction is not observed under treatment with BH4. **E**, There are no accumulations of CNP^+CC1^- immature oligodendrocytes after BH4 treatment during hypoxia. *P<0.05 vs hypoxia+BH4, †P<0.01 vs hypoxia+BH4, and P<0.05 vs normoxia by ANOVA with Bonferroni comparisons. Bar=50 µm. Data are shown as mean±SEM (n=5–8 each). BH4 indicates tetrahydrobiopterin; CNP, 2′,3-cyclic nucleotide 3′phosphodiesterase; DAPI, 4′,6-diamidino-2-phenylindole.

Consistent with previous observations,[44,47] MBP expression decreased with hypoxia as compared with normoxia (Figure 7A, 7B, 7D, and 7E). Treatment with BH4 eliminated the delayed myelination such that hypoxia with BH4 had increased MBP expression compared with hypoxia (Figure 7B through 7E). There was no difference in MBP expression between normoxia and hypoxia with BH4 (Figure 7A and 7C through 7E), thus suggesting that

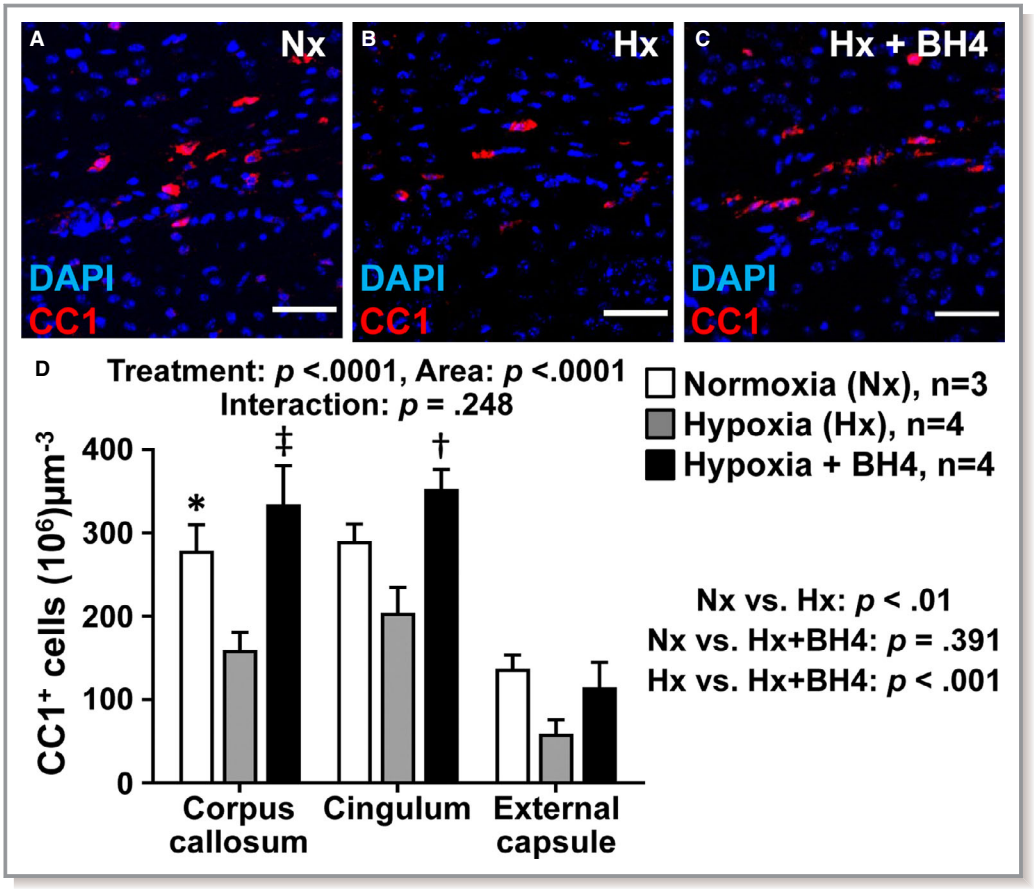

Figure 6. Benefits of BH4 treatment on hypoxia-induced arrested oligodendrocyte maturation persist in P30 mice. **A** through **C**, CC1+ mature oligodendrocytes in the corpus callosum among 3 groups. Bar=50 µm. **D**, Mature oligodendrocyte number is still decreased at P30 with hypoxia; the reduction is not observed under treatment with BH4. **P*<0.05, †*P*<0.01, and ‡*P*<0.001 vs hypoxia by 2-way ANOVA with Bonferroni comparisons. Data are shown as mean±SEM (n=3–4 each). BH4 indicates tetrahydrobiopterin; CNP, 2′,3-cyclic nucleotide 3′phosphodiesterase; DAPI, 4′,6-diamidino-2-phenylindole.

treatment with BH4 allows white matter oligodendrocytes not only to mature but also to myelinate under a chronic hypoxic condition.

Treatment Improves Long-Term Sensory-Motor Coordination Following Hypoxia in P30 Mice

Finally, we investigated sensory-motor coordination of P30 mice following hypoxic conditions, as fine and gross motor deficits are the most common neurologic deficits seen in children with CHD.[4,56] Using a 1-cm-wide inclined-beam walking task, sensory-motor coordination was impaired in hypoxia, as evidenced both by increased number of foot slips and longer traverse time (Figure 8A and 8B). Treatment with BH4 restored sensory-motor coordination such that number of foot slips and traverse time was no different between normoxia and hypoxia with BH4, thus suggesting that BH4 allows for normalization of motor abnormalities seen in hypoxia. There was no difference in task performance by sex.

Discussion

Using a mouse model of prenatal hypoxic white matter dysmaturation, our results support the hypothesis that BH4 levels are depleted in the developing brain under conditions of chronic hypoxia. Our results also support our hypothesis that treatment with BH4 under hypoxic conditions mitigates the toxic effects of chronic hypoxia on the developing brain. BH4 treatment under hypoxic conditions prevents increases in apoptosis of the developing white matter. Further, it does so without interfering with hypoxia-induced expansion of the oligodendrocyte pool. In oligodendrocyte lineages specifically, BH4 does not change apoptosis; rather, the treatment eliminates hypoxia-induced arrested oligodendrocyte maturation. Importantly, the improvement in oligodendrocyte maturation seen with BH4 treatment persists beyond the initial period of hypoxia. The present study also determines that BH4 replacement therapy eliminates delayed myelination attributable to chronic hypoxia. Finally, BH4 treatment causes

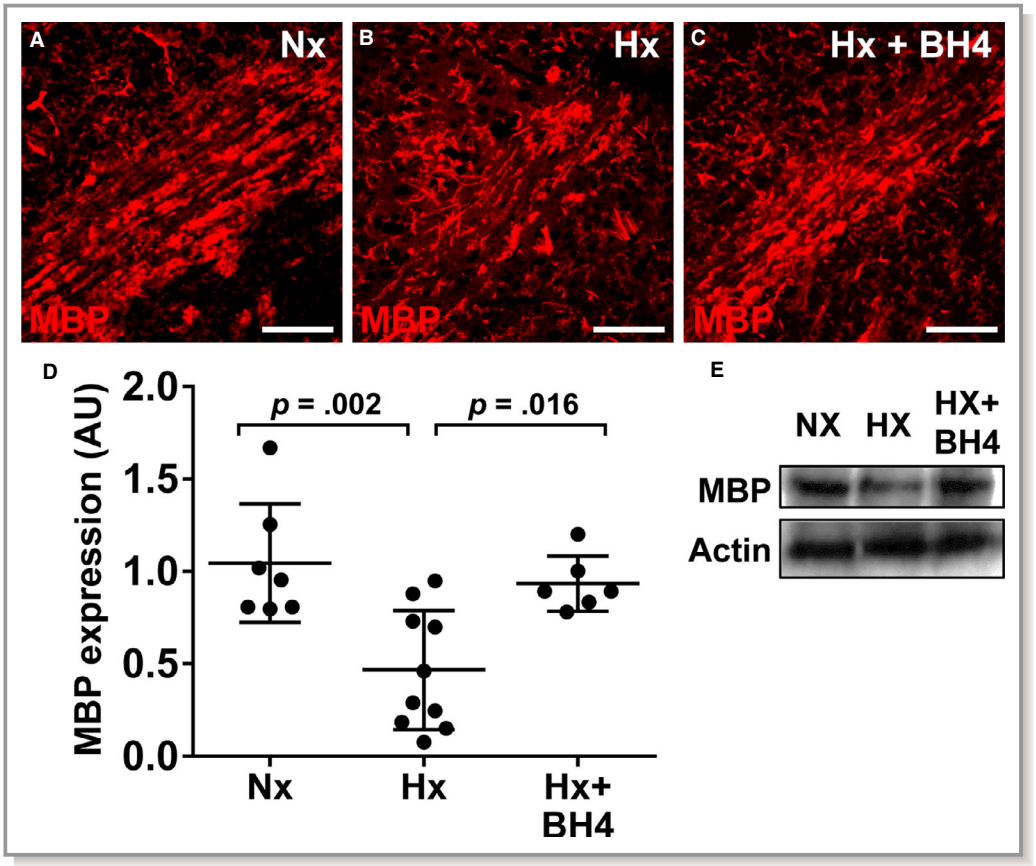

Figure 7. Treatment with BH4 eliminates delayed myelination resulting from chronic hypoxia. **A** through **C**, Myelin basic protein (MBP) expression in the corpus callosum among 3 groups. **D**, MBP expression level decreases following hypoxia. Treatment with BH4 results in increased expression compared with hypoxia. **E**, Western blot of white matter tissue in 3 groups. Actin size 42 kDa, MBP size 22 kDa. Bar=50 μm. Data are shown as mean±SEM (n=6–10 each). Hx indicates hypoxia; MBP, myelin basic protein; Nx, normoxia.

measurable and persistent improvements in sensory-motor coordination beyond the initial hypoxic period. All of these findings support the potential development of a new prenatal treatment to improve neurodevelopmental outcomes in the CHD population.

Multiple mouse models have been developed to mimic the complex cardiac structures of CHD.[57] However, nearly all genetic mouse models of complex CHD result in embryonic lethality; therefore, the pathologic effects of CHD on white matter maturation cannot be assessed in these models.[28] Unlike in humans, white matter development in the mouse brain begins postnatally.[58,59] Our mice are exposed to hypoxia during a period that corresponds to the human third trimester specifically for white matter development.[60] For the human fetus with CHD, the third trimester is particularly important for white matter development, as this is the period in which the greatest progressive white matter volume decline is seen in complex CHD.[49] Our mouse model has demonstrated similar phenotypes of white matter abnormalities as seen in human neonates with CHD, such as microstructural alterations determined by diffusion tensor imaging,[7,44] indicating that our mouse model uniquely replicates white matter pathology attributable to in utero hypoxia in the human fetus with CHD.

As survival from CHD increases, especially for complex disease such as transposition of the great arteries and single-ventricle lesions, neurologic sequelae are increasingly recognized. There are large personal, family, and societal costs of neurologic abnormalities in this growing community of patients. Neurologic insults in complex CHD start in utero,[7,61] and altered white matter microstructure from the perinatal period persists into adolescence.[18,62,63] Alterations in fetal cerebral oxygen delivery attributable to CHD lead to delayed brain maturation at birth.[64–67] Notably, immature white matter is at increased risk for further brain injury during cardiac surgery.[8,10] There are no effective treatment options currently available for neuroprotection of the fetus with CHD. Our results suggest that suboptimal BH4 levels influence hypoxic white matter derangements and that replacement therapy with BH4 improves hypoxia-induced delayed white matter maturation.

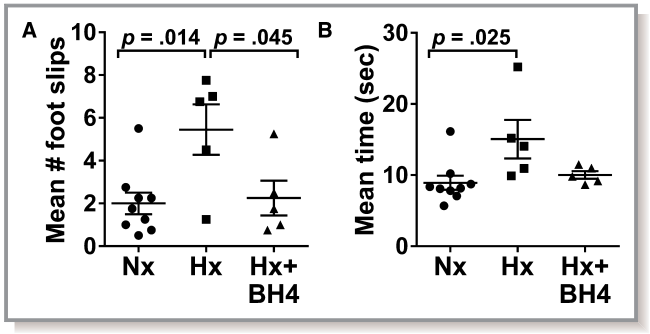

Figure 8. BH4 treatment improves long-term sensory-motor coordination following hypoxia in P30 mice. **A** and **B**, On the inclined beam-walking task (1 cm width), number of foot slips (**A**) and traverse time (**B**) increase with hypoxia, and this increase is not observed with BH4 treatment. Data are shown as mean±SEM (all groups n=5, except for Nx n=9). BH4 indicates tetrahydrobiopterin; Hx, hypoxia; Nx, normoxia.

A synthetic form of BH4 (sapropterin dihydrochloride) is already a Food and Drug Administration–approved treatment for phenylketonuria.[68] Extensive safety testing has revealed this medication to be safe during pregnancy.[69,70] Common side effects that occurred in at least 4% of participants included rhinorrhea and pharyngitis in both the adult and pediatric population. Other reported side effects did not differ from placebo. Anaphylaxis reactions have occurred, but there have been no other serious adverse effects in adult and pediatric populations.[71–73] A study of 16 pregnancies carried by women with phenylketonuria who were taking BH4 during gestation[69] revealed 13 full-term live births, 2 spontaneous abortions, and 1 preterm birth at 35 weeks' gestation. Maternal phenylketonuria is a known risk factor for spontaneous abortion, but the incidence of spontaneous abortion observed in the cohort was similar to the general population. The only offspring adverse event reported as possibly related to BH4 administration was hypophagia in the infant born preterm, and this resolved at 9 days of age. In addition to BH4's extensive safety record, it crosses the placenta and distributes throughout the fetus, including brain tissue.[74,75] There have been significant improvements in white matter integrity for human patients with phenylketonuria who were treated early with BH4.[76,77] As such, repurposing BH4 as a neuroprotective treatment for fetuses with CHD by providing it as a maternal medication following prenatal diagnosis is a feasible goal. The average prenatal age of CHD diagnosis in the United States is from the late second to early third trimester in the human fetus.[78] BH4 treatment in our animal model was started on postnatal day 3, equivalent to the early third trimester in humans (Figure 1C). Our current results take a translational approach toward developing a new maternal therapy aimed at reducing white matter immaturity in the fetus with CHD and white matter injury after neonatal cardiac surgery.

Limitations to our data include the inherent limitations of using an animal model to simulate human disease. Our study investigates the effects of hypoxia and BH4 treatment on the white matter development that occurs during the murine equivalent to the third trimester of human pregnancy. In mice, this period of white matter development begins in the postnatal period, and thus hypoxic rearing began on P3. There are important differences between the prenatal and postnatal environment, perhaps the most relevant being the increase in oxygen tension at birth to normal levels. By letting the mice develop normally until P3, it is possible that we are not accounting for developmental abnormalities that would have occurred before our selected developmental period. Another limitation of our animal model is that we had to approximate fractional inspired oxygen concentration to simulate the human condition. Magnetic resonance imaging studies of human fetuses with cyanotic CHD demonstrate a mean ascending aortic saturation of 48%.[26] Pulse oximetry in rodents breathing 10% fractional inspired oxygen concentration revealed arteriolar saturations between 55% and 70%,[79] and direct blood sampling of rodents breathing 12% fractional inspired oxygen concentration revealed a mean arterial oxyhemoglobin saturation of 53% with a partial pressure of oxygen of 41.5 mm Hg.[80] It is possible that the hypoxia experienced by the animals had a more severe effect than would be seen in human cyanotic CHD because of the differences between the prenatal and postnatal environments.

The etiology of poor neurodevelopmental outcomes in children with CHD is likely multifactorial. Recent large consortia studies have demonstrated striking overlap between genes in probands with CHD and autism,[81,82] suggesting a common genetic etiology of CHD and brain anomalies. Therefore, in situations in which genetic differences may drive brain abnormalities, those brain abnormalities may be less amenable to the proposed BH4 treatment. However, the cellular and molecular mechanisms whereby genetic alterations affect brain development in CHD are largely unexplored. Sophisticated genetic tools exist to allow investigations into the effects of hypoxia and/or BH4 treatment on mouse models of genetically manipulated brains. Thus, future studies with animal models will be essential to determining how genetic alterations identified in CHD patients affect the molecular and cellular programs underlying brain development, and this will be of great importance in establishing an optimal regimen for prenatal BH4 treatment in the fetus with CHD.

A combination of genetics, chronic hypoxia, and abnormal flow patterns could all contribute to the development of brain abnormalities in cyanotic CHD.[83] Our research focuses specifically on alleviating the harmful effects of

hypoxia on brain development. It is our hope that by reducing hypoxic brain injury in the cyanotic CHD population, neurodevelopmental outcomes will overall improve. The impact of this improvement is limited by the multifactorial nature of brain pathology in this population, and we emphasize the point that each possible etiology requires thorough investigation. Although immature white matter is more vulnerable to injury than mature white matter, it also retains a greater plasticity and more effective endogenous repair mechanisms, including remyelination and functional recovery of white matter axons by oligodendrocyte progenitors.[84,85] To attain successful white matter development in children with CHD, it will be critical to understand how the various factors contributing to brain pathology affect these endogenous repair mechanisms and developmental cellular processes.

The translation from bench science to clinical application is currently one of society's largest demands on the medical research community.[86,87] We recently established a chronic hypoxia model in newborn piglets that models impaired cortical development in human infants born with cyanotic CHD.[88] Our future studies using the large animal model, together with a pharmacokinetic approach, will assist in establishing an optimal regimen for maternal BH4 administration. Ultimately, we plan to move forward with designing a clinical trial for neuroprotection in the human population with cyanotic CHD.

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Disclosures

None.

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