Published in final edited form as:

J Pathol. 2012 February ; 226(3): 495–508. doi:10.1002/path.2980.

Mouse Intermittent Hypoxia Mimicking Apnea of Prematurity: Effects on Myelinogenesis and Axonal Maturation

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Abstract

Premature babies are at high risk for both infantile apnea and long-term neurobehavioral deficits. Recent studies suggest that diffuse structural changes in brain white matter are a positive predictor of poor cognitive outcomes. Since oligodendrocyte maturation, myelination, axon development and synapse formation mainly occur in the 3rd trimester of gestation and 1st postnatal year, infantile apnea could lead to and/or exaggerate white matter impairments in preterm neonates. Therefore, we investigated oligodendroglia and axon development in a neonatal mouse model of intermittent hypoxia between postnatal days 2 to 10. During critical phases of central nervous system development, intermittent hypoxia induced hypomyelination in the corpus callosum, striatum, fornix and cerebellum, but not the pons or spinal cord. Intermittent hypoxia-elicited alterations in myelin-forming processes were reflected by decreased expression of myelin proteins, including MBP, PLP, MAG and CNPase, possibly due to arrested maturation of oligodendrocytes. Ultra-structural abnormalities were apparent in the myelin sheath and axon. Immature oligodendrocytes were more vulnerable to neonatal intermittent hypoxia exposures than

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No conflicts of interest were declared

Statement of author contributions

JC and DG designed the study, interpreted the data, and coordinated the project. JC and CMT performed the animal treatments, morphological studies, and biochemical assays. YZ and CBS carried out tMMEP recording and analyzing. GG conducted the EM analysis. HF provided statistic analysis. The manuscript was written by JC, revised by CBS and DG. The submitted version was approved by all authors.

developing axons, suggesting that hypomyelination may contribute, at least partially, to axonal deficits. Insufficient neurofilament synthesis with anomalous components of neurofilament subunits, β -tubulin and MAP2 isoforms indicated immaturity of axons in intermittent hypoxia-exposed mouse brains. In addition, down-regulation of Synapsin I, Synaptophysin and Gap-43 phosphorylation suggested a potential stunt in axonogenesis and synaptogenesis. The region-selective and complex impairment in brain white matter induced by intermittent hypoxia was further associated with electrophysiological changes that may underlie long-term neurobehavioral sequelae.

Keywords

infantile apnea; intermittent hypoxia; white matter; oligodendrocyte; axon; brain

Introduction

Infantile apnea is a common condition in the newborn period, and its prevalence and severity increase as the level of maturity at birth decreases. More than 50% of premature infants and almost all infants weighing less than 1000 grams at birth suffer from recurrent apneic events during sleep (Finer et al., 2006), which are manifest as episodic hypoxemic events, with or without associated bradycardia. Along with such cardiorespiratory manifestations of apnea of prematurity (AOP), these infants are at higher risk for development of periventricular hemorrhagic infarction and periventricular leukomalacia, ultimately resulting in spastic motor deficits and long-term cognitive/behavioral impairments. Indeed, although survival of preterm infants without apparent brain injury has significantly improved due to advances in neonatal intensive care, long-term developmental sequelae are still a major concern (Als et al., 2004; Kleine et al., 2003; McCormick, 1997; Wilson-Costello et al., 2005; Wolke, 1998).

Compared with full-term infants, preterm children may exhibit prominent diffuse cerebral white matter impairments that underlie neurobehavioral deficits (Peterson et al. 2000; Inder et al., 2005). During the latter part of gestation and first postnatal months oligodendrocytes develop to form the myelin sheath surrounding axons. Axon maturation as well as synaptogenesis also occurs during the 3rd trimester to the 1st year after birth (Graaf-Peters and Hadders-Algra, 2006). Thus, AOP may affect these processes. Recently, extensive alterations in brain white matter were reported in adults with obstructive sleep apnea (Macey et al., 2008), and also in children suffering from congenital central hypoventilation syndrome (Kumar et al., 2008, 2010), strongly suggesting a link between sleep disordered breathing, white matter injury, and neurobehavioral dysfunction. The recurrent events of deoxygenation-reoxygenation that constitute one of the hallmarks of AOP could potentially lead to disrupted white matter formation. Indeed, brains of rats and mice exposed to chronic sustained hypoxia within the first postnatal weeks revealed decreased cortical volume and a paucity of subcortical white matter (Turner et al., 2003; Schwartz et al., 2004), which have been associated with developmental and behavioral deficits (Chahboune et al., 2009). However, it is not yet determined whether infantile apnea-associated intermittent hypoxia will result in developmental deficiency in CNS white matter.

In this study, we used a neonatal mouse model of intermittent hypoxia (IH) to simulate the hypoxia/reoxygenation events occurring in AOP (Cai et al., 2011). We hypothesized that apnea-associated IH could lead to white matter impairments seen in premature brain. The findings showed that immature oligodendrocytes were more vulnerable to IH exposures than developing axons during the neonatal period. The synthesis of myelin proteins in oligodendrocytes and of cytoskeletal proteins in neurons was inhibited and resulted in hypomyelination and abnormal ultra-structure of myelinated axons. Concomitantly, essential proteins related to axon maturation and synapse formation were altered as well. These morphological defects were associated with slow and attenuated conduction.

Materials and methods

Animals

All of the mice used in this study were handled according to protocols approved by the Institutional IACUC committee, as detailed in Supplemental Experimental Procedures.

Short-term neonatal IH exposures

Neonatal C57BL/6 P2 pups were culled for exposures. Eight pups were divided into two groups – intermittent hypoxia group (IH) and intermittent air control group (IA). The IH paradigm consisted of 20.9% O₂/8.0% O₂ alternation cycles (and in some cases 20.9% O₂/5.7% O₂ alternation cycles) lasting 120 s (i.e., 30 episodes per hour) for 6 hours a day during daylight from P2 to P10 (Supplemental Experimental Procedures). After IH exposures, pups were transferred to room air until euthanasia for tissue collection.

Quantitative reverse transcriptase PCR

Total RNA was isolated from whole mouse brains. Quantitative real-time PCR was performed on 7500 real-time PCR system (AB, Foster City, CA) using TaqMan One-step RT-PCR Master Mix Reagent (Supplemental Experimental Procedures).

Immunofluorescent staining

Single or double immunofluorescence on cryostat brain sections was performed as described previously (Cai et al., 2005). For the details of image acquisition and quantification, see Supplemental Experimental Procedures.

Western blotting

Protein samples from mouse brains were extracted in CelLytic™ MT Cell Lysis Reagent (Sigma) plus Complete Protease Inhibitors (Roche) at 4°C and immunoprecipitation was conducted using standard biochemical procedures (Supplemental Experimental Procedures).

BrdU labeling, proliferation rate and cell cycle exit index in oligodendrocyte progenitor cells

Bromodeoxyuridine (BrdU, 10µg/µl) was administered via peritoneal injections at either 1 or 24 hours before sacrifice. Brain was sectioned and stained. The proliferation rate and cell cycle exit index were calculated as described in Supplemental Experimental Procedures.

Electron microscopy

IH-insulted pups and their IA counterparts were perfused intracardially with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH7.2) at room temperature. Tissues were removed, postfixed in 1% osmium tetroxide in cacodylate buffer, and embedded in Embed812 epoxy resin. The micrographs from corpus callosum, striatum and optic nerve were captured under a Philips CM10 transmission electron microscope (Supplemental Experimental Procedures).

Electrophysiological evaluation - tcMMEP recording

The motor evoked potential elicited with transcranial magnetic stimulation (tcMMEP) is capable of monitoring the excitability of the brain and conduction properties of descending tracts of entire nervous system (Linden et al., 1999, Zhang et al., 2007). The recording of tcMMEP was detailed in Supplemental Experimental Procedures.

Statistical analyses

All data were shown as mean $\pm$ SD. Comparisons between IA and IH exposures were conducted using unpaired Student's *t* tests and effect sizes were calculated. Comparisons of different IH exposure days and different IH intensity were performed using ANOVA procedures and unpaired Student's *t* tests followed by the Bonferroni-Holm correction. Differences were considered as statistically significant for *p* values <0.05 .

Results

Apnea of prematurity-associated IH leads to region-selective hypomyelination in developing brain white matter

A previous study has reported that hypomyelination occurs in the corpus callosum after 4 weeks of continuous IH exposure (21% O₂/11% O₂ switch/ $\sim$ 10 min, 6 episodes per hour, 24 hours a day) in neonatal mice (Kanaan et al., 2006). In order to mimic hypoxia/reoxygenation events occurring in infantile apnea, we adopted a shorter IH/IA profile (2 min/cycle, 6 hrs/day, and 8 days). To evaluate if neonatal IH exposures affect myelin formation, we stained transverse and/or sagittal IH- and IA-treated brain sections with anti-MBP and/or anti-NF200 antibodies in the white matter areas of corpus callosum, external capsule, striatum, fornix, cerebellum, pons and spinal cord. Myelinated fibers in striatum, external capsule (Figure 1 A), fornix and cerebellum (Figure 1 B) were significantly reduced in P10 IH-exposed neonates, but not in pons and spinal cord (data not shown). Few myelinated fibers were observed in both IA- and IH-exposed corpora callosa (Figure 1 A, B). Twenty days later, after IH exposure was ended, percentages of myelinated area, as determined by immunostaining with anti-MAG antibody in corpus callosum and striatum, had not fully recovered in IH-exposed mice (Figure 1 C and D).

Alterations in myelin proteins and abnormal ultra-structure in IH-exposed neonatal mouse brain

The region-selective hypomyelination in the brain elicited by neonatal IH suggested a possible interference on oligodendrocyte development. Therefore, we examined the

expressions of myelin-relevant molecules at both transcriptional and translational levels. The differentiating and mature oligodendrocyte markers, including MBP, PLP, MAG, CNPase, and GalC, were all significantly reduced after 8 days neonatal IH exposures (Figure 2, supplemental Table 1). To further determine the extent of CNS myelination in neonatal IH-exposed brain, we examined the ultra-structural morphology of optic nerves, corpora callosa and striata by electron microscopy in P10 and P30 mouse brains. Within striatum and callosal areas, few axons were ensheathed in both IH- and IA-treated mice at P10, whereas optic fibers were myelinated sporadically and showed thinner myelin lamination in IH-exposed optic nerves (Cai et al., 2011). In contrast, most optic nerve, corpus callosum, and striatum axons formed compact myelin sheath at P30. In P30 IH-exposed brains, about 88% of axons in optic nerve (Figure 3 A) and 72% in striatum (Figure 3 B) were myelinated, compared to 93% and 79% in IA control brains. The *g*-ratio is a standard ultra-structural readout that provides a reliable index of myelination independent of axon diameter. The average *g*-ratios of optic nerve and striatum fibers of IH brains were significantly raised than those of IA-exposed neonates (Figure 3 C, supplemental Table 2). Intriguingly, IH did not change the axon diameter but affected myelin thickness in medium- to small-size axons (Figure 3 D, supplemental Table 2). No gross defects in the morphology of myelin sheath, paranode, or axon emerged in IH-exposed neonates. The high percentage of unmyelinated axons and axons with thinner myelin sheaths seen in neonatal brains after short-term IH exposure suggests that infantile apnea-associated IH may induce myelin dysgenesis during CNS development.

Inhibition of oligodendrocyte differentiation but not generation in IH-exposed neonatal mouse brain

To determine if decreased myelin-related proteins reflected an inhibited oligodendrogenesis or differentiation, we carried out fluorescent labeling with oligodendrocyte stage-specific markers Olig2, cytoplasmic Olig1, APC, and *pdgfra* in the corpus callosum in which myelination had not yet initiated. Olig2 is expressed across the oligodendrocyte lineage, from oligodendrocyte progenitor cells (OPCs) to mature oligodendrocytes (Lu et al., 2000; Takeyabashi et al., 2000; Zhou et al., 2000). Olig1 protein is localized in the nucleus in immature oligodendrocytes, but translocates to the cytoplasm in mature oligodendrocytes (Arnett et al., 2004; Cai et al., 2010; Kitada and Rowitch, 2006). At P10, fewer oligodendrocytes with strong cytoplasmic Olig1 staining were detected in the IH-exposed corpus callosa (Figure 4 A). Consistently, using CC1 antibody recognizing the APC protein expressed specifically in differentiated oligodendrocytes (Bhat et al., 1996), we found dramatically fewer APC⁺ oligodendrocytes in the corpus callosum and the adjacent region (Figure 4 B and D, white arrowheads). However, *pdgfra*⁺ OPCs (GFP⁺ cells) were increased but not Olig2⁺ cells in the same region (Figure 4 B–D). Congruously, expression of PDGFR α and NG2, two specific proteins co-localized with OPCs during early CNS development, was up-regulated in IH-insulted brain (Figure 4 E).

Altered differentiation of oligodendrocytes is associated with impaired cell cycle exit but not cell death in IH-exposed neonatal mouse brain

More oligodendrocyte progenitors, but fewer mature oligodendrocytes, may reflect the presence of OPC trapped in the cell cycle and/or cell death occurring in mature

oligodendrocytes. To test this hypothesis, we first examined the proliferation rate and cell cycle exit index in the corpus callosa of P10 IH- and IA-treated siblings. IH exposures did not alter the proportion of BrdU labeled cells significantly in OPCs (Figure 5 A). However, the cell cycle exit index was lower in IH-exposed brains (Figure 5 B).

To further rule out the possibility that a lack of mature oligodendrocytes in IH brain is due to the process of cell death, we examined the expression of Caspase-3. As shown in Figure 5 C, the reduction in mature oligodendrocytes was not associated with a detectable increase in apoptosis. Taken together, these findings suggest that IH elicits arrested maturation of OPCs in the developing brain.

Disrupted expression of neurofilament subunits, β -tubulin III and microtubule-associated proteins in IH-exposed neonatal mouse brain

Axons exhibit an intimate anatomic and functional relationship with myelinating oligodendrocytes. The interaction between oligodendrocytes and axons is critical for both oligodendroglial and axonal survival, maturation, and function. Hypomyelination in IH-insulted neonatal brains might be accompanied by deficits in axonal development. To investigate this possibility, we studied the cytoskeletal elements and related proteins in neurons. The number of neurofilaments (NFs) and their packing density regulated by phosphorylation of NF-M and NF-H subunits of the NF triplet directly determine the axonal diameter (Friede and Samorajski, 1970; Hoffman et al., 1985). Quantitative PCR showed that all transcripts of neurofilament components (NF-L, M, and H) were significantly decreased in P10 brains after 8-day IH exposure, in which NF-L and -M declined by approximately 20% while NF-H declined more than 30% (supplemental Table 1). The translation and phosphorylation of NF-H and -M were decreased as well (Figure 6 A). Consistently, expression of β -tubulin III, an assembling subunit for microtubules in neurons, was inhibited (Figure 6 B). IH exposure also impacted microtubule-associated proteins. The low molecular weight isoform MAP-2c was sustained at a lower level compared to the normal developing brain at P10 (Figure 6 C). However, total Tau and its phosphorylated forms at Ser396 and Thr231 were not changed in IH-exposed brains (Figure 6 D). All of these observations are indicative of axonal immaturity.

Inhibition on synapse-related proteins by IH in neonatal mouse brain

Recent studies revealed that synaptic formation, architecture and function are sensitive to changes in microtubule dynamics (Jaworski et al., 2009; Penzes et al., 2009). The disrupted neurofilament and microtubule composition associated with postnatal IH suggested that synaptic disturbances may be present as well. To test this hypothesis, we examined three synaptogenesis-relevant molecules – synaptic vesicle membrane proteins Synapsin I and Synaptophysin, and GAP-43. Both Synapsin I and Synaptophysin are localized in the nerve terminals of axons, which are considered as precise indicators of synapse formation. In IH-exposed neonatal brains, expression of Synapsin I and Synaptophysin declined at both transcriptional (Synapsin I: 0.79 ± 0.03 ; Synaptophysin: 0.84 ± 0.05 ; $n=9$, $p<0.01$) and translational levels (Figure 7 A). GAP-43 is abundant in axonal growth cones and presynaptic nerve terminals. Phosphorylation of GAP-43 inhibits GAP-43 from binding with Calmodulin and allows it to remain in an active status (Benowitz and Routtenberg, 1997) for

various intracellular functions such as axonal path finding, cytoskeletal organization at nerve endings, and synaptogenesis. We found that IH did not modify the expression of GAP-43 but down-regulated GAP-43 phosphorylation, which blunted GAP-43 activity (Figure 7 B).

Immature oligodendrocytes are more vulnerable to neonatal IH exposures than developing axons

We have heretofore shown that dysgenesis in brain white matter elicited by associated IH involved both oligodendrocyte progenitor arrest and disrupted axon maturation. Since oligodendrocytes and axons are interdependent during development, we aimed to identify whether oligodendrocytes or neurons constitute the primary target injury under IH insult. To answer this question, we implemented different paradigms for IH exposures on postnatal pups starting at P2 and evaluated the transcriptional changes of myelin-related molecules and neurofilament subunits using quantitative PCR (Figure 8, supplemental Table 1). The transcriptional decrease of *mbp*, *plp* and *MAG* was observed after 4 days IH exposure when the nadir fraction of inspired oxygen ($F_{I}O_2$) in chamber was 8% but did not affect NF subunits, indicating that immature oligodendrocytes are more vulnerable than developing axons in response to IH at molecular level. However, *mRNA* expression of neurofilament subunits decreased either after 8 days exposures with 8% nadir $F_{I}O_2$ or 4 days exposures using 5.7% nadir $F_{I}O_2$, suggesting a time- and dose-dependent effect of IH.

Electrophysiological evaluation in IH-insulted developing mice

To determine whether the heterotopic and complex lesions in brain elicited by neonatal IH impose functional consequences, we tested the tcMMEP that represents the efficacy of excitable signal transmission in the entire descending pathways (Figure 9 A). The onset latencies of tcMMEP in IH mice (5.12 ± 0.10 ms, $n=18$) were significantly increased compared to IA mice (4.98 ± 0.10 ms, $n=16$; $p<0.05$), while the amplitude was markedly reduced (IA: 2.34 ± 1.07 mV, $n=16$; IH: 0.67 ± 0.32 mV, $n=18$; $p<0.05$), indicating that electrical signals were attenuated when reaching the skeletal muscle in hind limbs (Figure 9B).

Discussion

The myelin-forming process occurs in the second week of postnatal development in rodents or the last trimester of gestation in human, and is not complete in some brain regions until adolescence/early adulthood (Rice and Barone 2000). An extensive body of evidence suggests that white matter structure is dynamic and contributes to normal information processing and learning. Disruption of white matter is associated with a wide range of cognitive and psychiatric disorders, indicating that myelin is indispensable for normal brain function (Fields, 2008). Therefore, identifying both extrinsic and intrinsic factors that modulate pathophysiological changes in white matter during CNS development is important not just for understanding developmental trajectories, but also for providing initial clues for preventative treatment of neurological and psychiatric diseases. In this study, we found that infantile apnea-associated IH stunts the development of brain white matter and leads to electrophysiological dysfunction, which is mediated, at least in part, by disturbance of myelinogenesis.

Neonatal IH induces multiregional complex impairments during CNS development

Previous studies reported that multifocal white matter lesions were found in preterm infants (El-Dib et al., 2010; Ment et al., 2009) and patients of congenital central hypoventilation syndrome (Kumar et al., 2008, 2010). In our mouse IH model of AOP, regions of defective myelin were unevenly distributed across multiple sites within the CNS (Figure 1 and 3, supplemental Table 2), suggesting both diffuse and focal impairments of white matter may be caused by the recurring deoxygenation-reoxygenation events. The OPCs were arrested and could not differentiate into mature oligodendrocytes (Figure 2, 4 and 5, supplemental Table 1), which may lose the optimal niche for myelin-forming process. Only a limited number of mature oligodendrocytes possessed a capability to wrap adjacent axons.

Beyond defects of oligodendrocytes and myelin structure, we found that white matter may also be affected by stunted axon growth in the IH-exposed neonatal brains. NFs promote axonal growth and determine axonal caliber. The compositional change of NF triplet subunits impacts both morphology and physiology of axons (Perrot et al., 2008; Walker et al., 2001). Tubulins (α and β) are synthesized and actively transported down the axon to assemble microtubules that regulate neuronal polarization, axon growth, remodeling of dendritic spines and transport of cargo molecules (Baas, 1997; Janke and Kneussel, 2010). Insufficient cytoskeleton components and low NF-H composition among NF triplet (Figure 6 A and B, supplemental Table 1) might result in a potential inhibition of filament assembly and radial growth of axons (Marszalek et al., 1996; Wong et al., 1995).

The microtubule-associated proteins, including MAP2 and Tau, are other important cytoskeletal molecules to regulate microtubule polymerization and stabilization, which promote neurite and axon outgrowth (Dehmelt and Halpain, 2005). In our study, we found that the low molecular weight isoform – MAP2C protein was dramatically decreased in neonatal IH-exposed brain while total MAP2 transcript and high molecular weight MAP2A/B were not affected (Figure 6 C). It has been reported that post-transcriptional and translational modifications play an important regulatory function on NF and tubulin expression (Janke and Kneussel, 2010; Szaro and Strong 2010). The lower level of MAP2C expression suggested that IH could activate a post-transcriptional regulation on RNA splicing and processing of MAP2 transcript. Additionally, our findings also alluded to an impairment of synaptic formation and function (Figure 7). Taken together, neonatal IH may cause region-selective and complex lesions in myelin, axon and synapse during CNS development (Figure 10).

Subtle but long-lasting CNS dysfunction following neonatal IH

“Low-risk” preterm infants are still at risk for higher morbidity, and exhibit increased odds for developing motor, cognitive, or behavioral disturbances (Majnemer et al., 1992; Mouradian et al., 2000), and to experience school difficulties (Chyi et al., 2008; Huddy et al., 2001, Myers and Ment LR, 2009). Follow-up *in vivo* imaging studies revealed diffuse low-density of white matter, punctate white matter lesions, and excessive high signal intensity in broad spectrum of brain regions (Als et al., 2004; El-Dib et al., 2010; Rutherford et al., 2010). Our findings in mice support the notion that neonatal IH contributes to multiregional white matter lesions and leads to long-lasting adverse sequelae in some brain

areas such as corpus callosum. We should remark that the neonatal IH-exposed mice exhibited seemingly normal growth somatic and motor growth as their IA-treated littermates. We did not observe a difference in body or brain weight, and laminar structure of cerebral cortex after short-term IH exposure (Cai et al., 2011; data not shown). However, the excitability of the brain or the conduction of electric signals released from cortical neurons was significantly delayed and attenuated along the corticospinal tract even after IH insult was withdrawn for several weeks (Figure 9). Clinical studies reported that myelin insufficiency or breakdown during the myelinating process is associated with the onset of psychiatric diseases and Alzheimer's disease later in life (Bartzokis et al., 2006, 2007; Davis et al., 2003; Tkachev et al., 2003; Zai et al., 2005). The lack of a recovery of white matter lesion elicited by neonatal IH insult underlies the electrophysiological dysfunction and may constitute the etiology of other neurological or psychiatric diseases.

Mechanisms underlying the malmyelinogenesis associated with IH insult

The pathological consequences in premature infants have been found from focal cystic necrosis (periventricular leukomalacia) to multifocal or diffuse myelin disruption, which is caused by ischemic hypoxia in neural tissue. In contrast with ischemic hypoxia, neonatal apnea-associated IH is characterized by recurrent events of milder deoxygenation followed by reoxygenation. Albeit similar pathological consequences of disrupted white matter and synapse were observed in both sustained and intermittent hypoxia (Curristin et al., 2002), these consequences might be mediated by different pathological mechanisms. It has been reported that chronic sustained hypoxia and chronic intermittent hypoxia induced differential gene activation in either *in vitro* cell culture or *in vivo* rodent model of hypoxia (Ryan et al., 2005; Zhou et al., 2008). In our studies, we found that postnatal IH impeded oligodendrocyte progenitors to exit cell cycle and differentiate (Figure 5 B), but did not elicit a diffusive reduction of OPCs, as observed in the chronic sustained hypoxia mouse model (Back et al., 2006; Curristin et al., 2002). More OPCs in neonatal IH-exposed brain did not involve more OPC generation or hyper-proliferation, at least in callosal areas (Figure 4 D, E and Figure 5 A), suggesting that IH may activate different signaling pathways on hypoxia-induced white matter injury.

In the CNS, oligodendrocyte maturation and myelination is not synchronous across brain regions. For example, the differentiating oligodendrocytes are first present in mouse hindbrain at e14.5 and then found in pons and cervical spinal cord at e16.5 (Hardy and Friedrich 1996), i.e., 6 to 8 days earlier than that in the corpus callosum or striatum (Kim et al., 2008). These lags could explain why myelination occurred normally in IH-insulted pons and cervical spinal cords but not in corpus callosum, striatum, fornix or optic nerve (Figures 1 and 3, data not shown), as the maturation of oligodendrocytes might be almost completed in those areas when mice were exposed to IH. Since axon growth and synapse formation are also major neurobiological events occurring during the initial postnatal period in rodents, neonatal IH resulted as expected, in axonal and synaptic impairments in the developing mouse brain (Figures 6 and 7). Thus, depending on when IH develops according to the different timing windows of CNS development, we should anticipate different patterns of injury associated with IH.

The different regions and cell types in brain require different levels of oxygen delivery, and show variable sensitivity to hypoxia (Acker, 2004; Erecinska and Silver, 2001). IH is one of most prevalent causes of systemic oxygen deprivation and consequently of neurological end-organ dysfunction. We found that oligodendrocytes are more vulnerable to IH than axons, with the extent of white matter lesion(s) being dependent on the intensity and duration of IH. The more severe deoxygenation and longer hypoxia exposure, the greater and more extensive the damage will be (Figure 8, supplemental Table 1).

IH and sleep disturbance are the two predominant features of AOP and in this context are similar to sleep apnea in adults. The sleep-wake cycles and sleep states also influence breathing profoundly and may compound IH-induced pathophysiologic consequences. Thus, incorporation of sleep studies in our neonatal IH model and implementation of gas-exchange abnormalities in a sleep state selective manner would reveal more precise portraits of the alterations induced by sleep disordered breathing in the developing brain. However, polysomnographic recordings are not currently feasible on neonatal pups. Furthermore, changes in blood pressure and cerebral perfusion were not assessed in this model, and it is very likely that alterations in cerebral blood flow autoregulatory mechanisms may also be induced by intermittent hypoxia, and thus contributes to the findings presented herein.

In summary, the murine IH model of AOP clearly demonstrates that the episodic hypoxic events that characterize this condition can induce substantial dysgenesis of white matter during the critical phases of CNS development, suggesting that infantile apnea could cause functional impairments of brain white matter and long-term neurobehavioral sequelae, and that the latter may underlie the pathological basis of other neurological or psychiatric diseases later in life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to Drs. Chuck Stiles and John Alberta for anti-Olig1 and Olig2 antibodies. We thank the analytical microscopy core facility for providing access to EM instruments, and in particular Cathie Caple and Arkadiusz Slusarczyk for technical assistance. We also thank Dr. Nicholas Mellen for a critical reading of the manuscript and Dr. Robert M. Greene for the substantial support via COBRE program. This work is supported by NIH 2P20RR017702-061A1 (R.M.G., J.C. is a COBRE supported junior faculty and co-investigator), Sleep Research Society Foundation/J. Christian Gillin M.D. Research Grant (J.C.), University of Louisville SOM Basic Grant (J.C.), and NIH HL-086662 (D.G.).

List of abbreviations

AOP	apnea of prematurity
IH	intermittent hypoxia
IA	intermittent air
F_IO₂	fraction of inspired oxygen
CNS	central nervous system

OPC	oligodendrocyte progenitor cell
Olig1/2	oligodendrocyte transcription factor 1/2
pdgfra	platelet-derived growth factor receptor alpha
NG2	NG2 chondroitin sulfate proteoglycan
MBP	myelin basic protein
PLP	proteolipid protein
MAG	myelin associated glycoprotein
GalC	galactosylceramidase
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
APC	adenomatous polyposis coli
NF-L/M/H	neurofilament light/medium/heavy subunit
MAP2	microtubule-associated protein 2
Gap-43	growth associated protein 43

References

1. Acker T, Acker H. Cellular oxygen sensing need in CNS function: physiological and pathological implications. *J Exp Biol.* 2004; 207:3171–3188. [PubMed: 15299039]
2. Als H, Duffy FH, McAnulty GB, et al. Early experience alters brain function and structure. *Pediatrics.* 2004; 113(4):846–57. [PubMed: 15060237]
3. Arnett HA, Fancy SPJ, Alberta JA, et al. bHLH Transcription Factor Olig1 Is Required to Repair Demyelinated Lesions in the CNS. *Science.* 2004; 306:2111–2115. [PubMed: 15604411]
4. Back SA, Craig A, Luo AL, et al. Protective Effects of Caffeine on Chronic Hypoxia-Induced Perinatal White Matter Injury. *Annal Neurol.* 2006; 60(6):696–705. [PubMed: 17044013]
5. Bartzokis G, Lu PH, Geschwind DH, et al. Apolipoprotein E Genotype and Age-Related Myelin Breakdown in Healthy Individuals: Implications for Cognitive Decline and Dementia. *Arch Gen Psychiatry.* 2006; 63:63–72. [PubMed: 16389198]
6. Bartzokis G, Lu PH, Geschwind DH, et al. Apolipoprotein E Affects Both Myelin Breakdown and Cognition: Implications for Age-Related Trajectories of Decline Into Dementia. *Biol Psych.* 2007; 62:1380–1387.
7. Baas PW. Microtubules and axonal growth. *Curr Opin Cell Biol.* 1997; 9(1):29–36. [PubMed: 9013665]
8. Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci.* 1997; 20:84–91. [PubMed: 9023877]
9. Bhat RV, Axt KJ, Fosnaugh JS, et al. Expression of the APC tumor suppressor protein in oligodendroglia. *Glia.* 1996; 17(2):169–74. [PubMed: 8776583]
10. Cai J, Tuong C, Gozal D. A neonatal mouse model of intermittent hypoxia associated with features of apnea in premature infants. *Respir Physiol Neurobiol.* 2011; 10.1016/j.resp.2011.06.003
11. Cai J, Qi Y, Hu X, et al. Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. *Neuron.* 2005; 45:41–53. [PubMed: 15629701]
12. Cai J, Zhu Q, Zheng K, et al. Co-localization of Nkx6.2 and Nkx2. 2 homeodomain proteins in differentiated myelinating oligodendrocytes. *Glia.* 2010; 58(4):458–68. [PubMed: 19780200]

13. Chahboune H, Ment LR, Stewart WB, et al. Hypoxic injury during neonatal development in murine brain: correlation between in vivo DTI findings and behavioral assessment. *Cerebral Cortex*. 2009; 19:2891–2901. [PubMed: 19380380]
14. Chyi LJ, Lee HC, Hintz SR, et al. School outcomes of late preterm infants: special needs and challenges for infants born at 32 to 36 weeks gestation. *J Pediatr*. 2008; 153:25–31. [PubMed: 18571530]
15. Curristin SM, Cao A, Stewart WB, et al. Disrupted synaptic development in the hypoxic newborn brain. *Proc Natl Acad Sci USA*. 2002; 99(24):15729–34. [PubMed: 12438650]
16. Davis KL, Haroutunian V. Global expression-profiling studies and oligodendrocyte dysfunction in schizophrenia and bipolar disorder. *Lancet*. 2003; 362:758. [PubMed: 13678867]
17. Dehmelt L, Halpain S. The MAP2/Tau family of microtubule-associated proteins. *Genome Biol*. 2005; 6(1):204. [PubMed: 15642108]
18. El-Dib M, Massaro AN, Bulas D, et al. Neuroimaging and Neurodevelopmental Outcome of Premature Infants. *Am J Perinatol*. 2010 Epub ahead of print.
19. Erecinska M, Silver IA. Tissue oxygen tension and brain sensitivity to hypoxia. *Respir Physiol*. 2001; 128:263–276. [PubMed: 11718758]
20. Fields RD. White matter in learning, cognition and psychiatric disorders. *Trends Neurosci*. 2008; 31(7):361–70. [PubMed: 18538868]
21. Finer NN, Higgins R, Kattwinkel J, et al. Summary proceedings from the apnea-of-prematurity group. *Pediatrics*. 2006; 117:S47–51. [PubMed: 16777822]
22. Friede RL, Samorajski T. Axon caliber related to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. *Anat Rec*. 1970; 167:379–388. [PubMed: 5454590]
23. De Graaf-Peters VB, Hadders-Algra M. Ontogeny of the human central nervous system: what is happening when? *Early Hum Dev*. 2006; 82:257–286. [PubMed: 16360292]
24. Hardy RJ, Friedrich VL. Oligodendrocyte progenitors are generated throughout the embryonic mouse brain, but differentiate in restricted foci. *Development*. 1996; 122:2059–2069. [PubMed: 8681787]
25. Hoffman P, Griffin J, Gold B, et al. Slowing of neurofilament transport and the radial growth of developing nerve fibers. *J Neurosci*. 1985; 5:2920–2929. [PubMed: 2414416]
26. Huddy CL, Johnson A, Hope PL. Educational and behavioural problems in babies of 32–35 weeks gestation. *Arch Dis Child Fetal Neonatal Ed*. 2001; 85:F23–F28. [PubMed: 11420317]
27. Inder T, Neil J, Kroenke C, et al. Investigation of cerebral development and injury in the prematurely born primate by magnetic resonance imaging and histopathology. *Dev Neurosci*. 2005; 27:100–111. [PubMed: 16046843]
28. Janke C, Kneussel M. Tubulin post-translational modifications: encoding functions on the neuronal microtubule cytoskeleton. *Trends Neurosci*. 2010; 33(8):362–72. [PubMed: 20541813]
29. Jaworski J, Kapitein LC, Gouveia SM, et al. Dynamic microtubules regulate spine morphology and synaptic plasticity. *Neuron*. 2009; 61:85–100. [PubMed: 19146815]
30. Kanaan A, Farahani R, Douglas RM, et al. Effect of chronic continuous or intermittent hypoxia and reoxygenation on cerebral capillary density and myelination. *Am J Physiol Regul Integr Comp Physiol*. 2006; 290:R1105–1114. [PubMed: 16322350]
31. Kim EJ, Battiste J, Nakagawa Y, et al. Ascl1 (Mash1) lineage cells contribute to discrete cell populations in CNS architecture. *Mol Cell Neurosci*. 2008; 38(4):595–606. [PubMed: 18585058]
32. Kitada M, Rowitch DH. Transcription factor co-expression patterns indicate heterogeneity of oligodendroglial subpopulations in adult spinal cord. *Glia*. 2006; 54(1):35–46. [PubMed: 16673374]
33. de Kleine MJ, den Ouden AL, Kollée LA, et al. Development and evaluation of a follow up assessment of preterm infants at 5 years of age. *Arch Dis Child*. 2003; 88(10):870–5. [PubMed: 14500304]
34. Kumar R, Macey PM, Woo MA, et al. Diffusion tensor imaging demonstrates brainstem and cerebellar abnormalities in congenital central hypoventilation syndrome. *Pediatr Res*. 2008; 64(3): 275–80. [PubMed: 18458651]

35. Kumar R, Macey PM, Woo MA, et al. Rostral brain axonal injury in congenital central hypoventilation syndrome. *J Neurosci Res*. 2010; 88(10):2146–54. [PubMed: 20209631]
36. Linden RD, Zhang YP, Burke DA, et al. Magnetic motor evoked potential monitoring in the rat. *J Neurosurg*. 1999; (Suppl):205–210. [PubMed: 10505506]
37. Lu QR, Yuk D-i, Alberta JA, et al. Sonic Hedgehog-Regulated Oligodendrocyte Lineage Genes Encoding bHLH Proteins in the Mammalian Central Nervous System. *Neuron*. 2000; 25:317–329. [PubMed: 10719888]
38. Macey PM, Kumar R, Woo MA, et al. Brain structural changes in obstructive sleep apnea. *Sleep*. 2008; 31(7):967–77. [PubMed: 18652092]
39. Majnemer A, Brownstein A, Kadanoff R, et al. A comparison of neurobehavioral performance of healthy term and low-risk preterm infants at term. *Dev Med Child Neurol*. 1992; 34(5):417–24. [PubMed: 1592194]
40. Marszalek JR, Williamson TL, Lee MK, et al. Neurofilament subunit NF-H modulates axonal diameter by selectively slowing neurofilament transport. *J Cell Biol*. 1996; 135(3):711–24. [PubMed: 8909545]
41. McCormick MC. The outcomes of very low birth weight infants: are we asking the right questions? *Pediatrics*. 1997; 99(6):869–76. [PubMed: 9164781]
42. Ment LR, Hirtz D, Hüppi PS. Imaging biomarkers of outcome in the developing preterm brain. *Lancet Neurol*. 2009; 8(11):1042–55. [PubMed: 19800293]
43. Mouradian LE, Als H, Coster WJ. Neurobehavioral functioning of healthy preterm infants of varying gestational ages. *J Dev Behav Pediatr*. 2000; 21(6):408–16. [PubMed: 11132791]
44. Myers E, Ment LR. Long-term outcome of preterm infants and the role of neuroimaging. *Clin Perinatol*. 2009; 36(4):773–89. [PubMed: 19944835]
45. Penzes P, Srivastava DP, Woolfrey KV. Not just actin? A role for dynamic microtubules in dendritic spines. *Neuron*. 2009; 61:3–5. [PubMed: 19146807]
46. Perrot R, Berges R, Bocquet A, et al. Review of the multiple aspects of neurofilament functions and their possible contribution to neurodegeneration. *Mol Neurobiol*. 2008; 38:27–65. [PubMed: 18649148]
47. Peterson BS, Vohr B, Staib LH, et al. Regional brain volume abnormalities and long-term cognitive outcome in preterm infants. *JAMA*. 2000; 284:1939–1947. [PubMed: 11035890]
48. Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect*. 2000; 108 (Suppl 3):511–33. [PubMed: 10852851]
49. Rutherford MA, Supramaniam V, Ederies A, et al. Magnetic resonance imaging of white matter diseases of prematurity. *Neuroradiology*. 2010; 52(6):505–21. [PubMed: 20422407]
50. Ryan S, Taylor CT, McNicholas WT. Selective activation of inflammatory pathways by intermittent hypoxia in obstructive sleep apnea syndrome. *Circulation*. 2005; 112:2660–2667. [PubMed: 16246965]
51. Schwartz ML, Vaccarino F, Chacon M, et al. Chronic neonatal hypoxia leads to long term decreases in the volume and cell number of the rat cerebral cortex. *Semin Perinatol*. 2004; 28:379–388. [PubMed: 15693394]
52. Szaro BG, Strong MJ. Post-transcriptional control of neurofilaments: New roles in development, regeneration and neurodegenerative disease. *Trends Neurosci*. 2010; 33(1):27–37. [PubMed: 19906448]
53. Takebayashi H, Yoshida S, Sugimori M, et al. Dynamic expression of basic helix-loop-helix Olig family members: implication of Olig2 in neuron and oligodendrocyte differentiation and identification of a new member, Olig3. *Mech Dev*. 2000; 99:143–148. [PubMed: 11091082]
54. Tkachev D, Mimmack ML, Ryan MM, et al. Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. *Lancet*. 2003; 362:798–805. [PubMed: 13678875]
55. Turner CP, Seli M, Ment L, et al. A1 adenosine receptors mediate hypoxia-induced ventriculomegaly. *Proc Natl Acad Sci USA*. 2003; 100:11718–11722. [PubMed: 12975523]
56. Walker KL, Yoo HK, Undamatla J, et al. Loss of neurofilaments alters axonal growth dynamics. *J Neurosci*. 2001; 21:9655–9666. [PubMed: 11739575]

57. Wilson-Costello D, Fridedman H, Minich N, et al. Improved survival rates with increased neurodevelopmental disability for extremely low birth weight infants in the 1990s. *Pediatrics*. 2005; 115:997–1003. [PubMed: 15805376]
58. Wolke D. Psychological development of prematurely born children. *Arch Dis Child*. 1998; 78(6): 567–70. [PubMed: 9713018]
59. Wong PC, Marszalek J, Crawford TO, et al. Increasing neurofilament subunit NF-M expression reduces axonal NF-H, inhibits radial growth, and results in neurofilamentous accumulation in motor neurons. *J Cell Biol*. 1995; 130(6):1413–22. [PubMed: 7559762]
60. Zai G, King N, Wigg K, et al. Genetic study of the myelin oligodendrocyte glycoprotein (MOG) gene in schizophrenia. *Genes, Brain and Behavior*. 2005; 4:2–9.
61. Zhang YP, Shields LB, Zhang Y, et al. Use of magnetic stimulation to elicit motor evoked potentials, somatosensory evoked potentials, and H-reflexes in non-sedated rodents. *J Neurosci Methods*. 2007; 165(1):9–17. [PubMed: 17628688]
62. Zhou D, Wang J, Zapala MA, et al. Gene expression in mouse brain following chronic hypoxia: role of sarcospan in glial cell death. *Physiol Genomics*. 2008; 32(3):370–9. [PubMed: 18056785]
63. Zhou Q, Wang S, Anderson DJ. Identification of a Novel Family of Oligodendrocyte Lineage-Specific Basic Helix-Loop-Helix Transcription Factors. *Neuron*. 2000; 25:331–343. [PubMed: 10719889]

Figure 1.

Immunostaining with anti-MBP (A), anti-NF200 and anti-MBP (B), and anti-MAG (C) antibodies on P10 (A–B) and P31 (C) transverse (A, C) or sagittal (B) brain sections. (D) Percentages of myelinated area determined by immunostaining with anti-MAG antibody in corpus callosum ($d=7.2$) and striatum ($d=6.1$) of IA- and IH-exposed mice. IA: intermittent air; IH: intermittent hypoxia. CC: corpus callosum, broken line; EC: external capsule; St: striatum; Cbl: cerebellum. Arrows indicate the myelinated regions. Scale bar: 100 μ m. Error bar: standard deviation. d : effect size. * represents $p < 0.01$ (n=4 mice).

Figure 2.

Expression of myelin-relevant molecules in P10 whole brains after 8-day IH of 8% nadir F_1O_2 and IA exposures. **(A)** Relative mRNA fold changes of *mbp*, *plp* and *MAG*. * indicates $p < 0.01$ (n=9 mice). **(B)** Representative Western blot analysis of MBP, PLP, MAG, CNP and GalC. Both transcripts and translations of myelin-relevant genes were significantly decreased in neonatal IH insulted brains.

Figure 3.

Representative electron micrographs of myelin ultra-structure in P30 IA- and IH-exposed brains. The transverse sections for the EM examination were prepared from optic nerve (**A**) and striatum (**B**). (**C**) Scatter plots of g-ratios against axon diameters calculated from 120 fibers in IA- (black) and IH-exposed (red) optic nerves and striatal areas. (**D**) Axon diameter and g-ratio of fibers grouped by axon diameter in IA- and IH-exposed groups. Error bar: standard deviation. * represents $p < 0.01$.

Figure 4.

Delayed differentiation of oligodendrocytes in corpus callosum of P10 brains undergoing 8 days neonatal IH. Transverse (A) and sagittal (B, C) forebrain sections through corpus callosum regions were prepared from IH-/IA-exposed wild-type mice (A, B) or *pdgfra-EGFP* transgenic mice (C). Olig2+ oligodendroglia lineage cells ($d=0.6$), APC+ mature oligodendrocytes ($d=1.8$), and *pdgfra*+GFP+ OPCs ($d=1.2$) were counted and calculated in per square millimeter (D). Expression of PDGFRα and NG2 was detected by whole-brain Western blot (E). CC: corpus callosum; SCC: splenium corpus callosum; GCC: genu corpus callosum. Dotted lines demarcate the CC areas. White arrowheads represented APC+ cells.

Scale bar: 100 μm . Error bar: standard deviation. *d*: effect size. ** indicates $p < 0.01$, * indicates $p < 0.05$ (n=8 mice).

Figure 5.

Neonatal IH does not affect the ability of OPC to proliferate, but arrests OPCs from exiting the cell cycle. **(A)** Representative images of immunohistochemistry with BrdU (red), GFP (green, *pdgfra*⁺ cells), and Dapi (blue). A similar percentage of BrdU⁺ cells among *pdgfra*⁺ OPCs was detected in both IA and IH-exposed corpora callosa ($d=0.9$). **(B)** Representative images of double immunostaining with antibodies against Ki-67 (green) and BrdU (red) revealed a lower cell cycle exit index in IH-insulted corpus callosa ($d=2.3$). **(C)** Western blotting with anti-Caspase3 to detect the cell death. Scale bar: 100 μ m. Error bar: standard deviation. d : effect size. * indicates $p<0.05$ ($n=4$ mice).

Figure 6.

Disrupted expression of cytoskeletal molecules and microtubule-associated proteins in P10 brain neurons elicited by 8-days of neonatal IH. **(A)** Expression of NF subunits in whole brain and immunostaining with anti-NF-H/M antibody in forebrain area. Error bar: standard deviation. * indicates $p < 0.01$; **(B)** The synthesis of β -tubulin protein; **(C)** and **(D)** Expression of microtubule-associated protein MAP2 and Tau.

Figure 7.

Expression of synaptic vesicle membrane proteins and phosphorylation of GAP-43 in P10 whole brains after 8-day IA of 8% nadir F_1O_2 or IH exposures. **(A)** Expression of Synaptophysin ($d=4.5$) and Synapsin I ($d=9.9$); **(B)** Expression of total GAP-43 protein and its phosphorylation. Error bar: standard deviation. d : effect size. * indicates $p<0.01$ ($n=9$ mice).

Figure 8.

Relative *mRNA* fold changes of myelin-related proteins and neurofilament subunits in whole brains after 4- and 8-day IA or IH exposures starting at P2. (A) 8% nadir F₁O₂; (B) 5.7% nadir F₁O₂. Error bar: standard deviation. * indicates $p < 0.01$ (n=9 mice).

Figure 9.

Electrophysiological changes in the descending tracts of entire nervous system in P31 young adult mice after 8-day IH exposures under 8% nadir F_{IO_2} from P2 to P10 ($n=16$ or 18 mice for IA or IH exposures). (A) Diagrammatic illustration of tcMMEP. (B) tcMMEP latency ($d=1.4$) and amplitude ($d=2.1$). Error bar: standard deviation. d : effect size. *** indicates $p<0.01$.

Figure 10.

A hypothetical hierarchy of white matter injury in AOP. SA, sleep apnea; OPC, oligodendrocyte progenitor cell; OL, oligodendrocyte; WM, white matter.