Differential Susceptibility to Axonopathy in Necrotic and Non-Necrotic Perinatal White Matter Injury

Art Riddle, PhD; Jennifer Maire, BS; Xi Gong, MD; Kevin X. Chen, BS; Christopher D. Kroenke, PhD; A. Roger Hohimer, PhD; Stephen A. Back, MD, PhD

Background and Purpose—White matter injury (WMI) is the leading cause of brain injury in preterm survivors and results in myelination failure. Although axonal degeneration occurs in necrotic lesions, the role of axonopathy in myelination failure remains controversial for diffuse non-necrotic WMI, which is currently the major form of WMI. We determined the burden of axonopathy in diffuse lesions.

Methods—We analyzed WMI in a preterm fetal sheep model of global cerebral ischemia that replicates the relative burden of necrotic and non-necrotic human WMI. WMI was analyzed at 1 or 2 weeks after ischemia and identified by ex vivo high-field (11.7 Tesla) magnetic resonance imaging of fixed brain tissue. Axonal integrity was analyzed by immunohistochemical detection of axon injury markers and by transmission electron microscopy to quantify axon loss and degeneration in magnetic resonance imaging-defined lesions.

Results—Axonal degeneration, defined by staining for neurofilament protein and β-amyloid precursor protein, was restricted to discrete necrotic foci with robust microglial activation. Unexpectedly, axonal degeneration was not visualized in the major form of WMI, which comprised large non-necrotic lesions with diffuse reactive astrogliosis. In these major lesions, quantitative electron microscopy studies confirmed no significant differences in the density of intact and degenerating axons or in the distribution of axon diameters relative to controls.

Conclusions—The mechanism of myelination failure differs significantly in perinatal WMI dependent on the burden of necrosis. Axonopathy is associated with focal necrotic injury but not with primary diffuse non-necrotic lesions, which supports that intact axons in the primary lesions are potential targets for myelination.

Key Words: axonal injury ■ electron microscopy ■ hypoxia-ischemia ■ prematurity ■ white matter injury

Cerebral hypoxia-ischemia is a common cause of white matter injury (WMI) in the developing brain and is a leading cause of life-long neurological disability in survivors of prematurity birth and infants with congenital heart disease. WMI is now the most common lesion in children with cerebral palsy and manifests as nonprogressive motor deficits and cognitive/learning disabilities. Advances in neuroimaging have identified a shift from predominantly large necrotic WMI (periventricular leukomalacia) to focal or diffuse nondestractive lesions. However, the burden of small foci of microscopic necrosis, which are poorly defined by MRI, was recently found to be low in human and in a preclinical model of WMI in fetal sheep.

The propensity for myelination failure distinguishes WMI from other forms of cerebral palsy that involve gray matter injury. In necrotic lesions, myelination failure arises from acute degeneration of axons and glia. Because of the pronounced reduction in necrotic WMI in contemporary cases, there has been increased study of myelination failure in non-necrotic lesions where diffuse astrogliosis predominates. One emerging mechanism of myelination failure in non-necrotic lesions involves disrupted maturation of the oligodendrocyte (OL) lineage. Although hypoxia-ischemia triggers substantial degeneration of late oligodendrocyte progenitors (pre-OL) during acute WMI, pre-OLs mount a robust regenerative response but fail to differentiate in chronic lesions.

Presently controversial, however, is the extent to which axonopathy contributes to myelination failure in these non-necrotic lesions and lesions with microscopic necrosis.

We addressed here the role of axonopathy in diffuse WMI because it has significant implications for therapeutic strategies to promote myelination in chronic WMI. We used a fetal sheep model of global cerebral ischemia in which we recently defined the relative burden of focal microscopic necrosis and diffuse WMI through a combination of histopathology and high-field MRI. Both forms of WMI involve variable de-
grees of astrogial and microglial activation. Because reactive glia can promote chronic inflammation and the generation of factors deleterious to axonal survival, we tested the hypothesis that the burden of axonopathy would be greater in necrotic lesions with mixed glial reaction than in non-necrotic diffuse WMI with predominantly astroglial reaction. We undertook the first quantitative ultrastructural studies to define axonal integrity in diffuse WMI identified by high-field MRI. Axonal degeneration was restricted to discrete foci of microscopic necrosis with pan-cellular loss, and significant axonopathy was not detected within large lesions with diffuse WMI. Hence, perinatal WMI displays differential susceptibility to axonopathy that is related to the burden of necrosis.

Materials and Methods

Animal Surgery
Surgery was performed on time-bred sheep of mixed western breed between 88 and 91 days of gestation (term, 145 days), modified from a previously described protocol. For detailed methods on surgical procedures, physiological monitoring, and blood analyses, please see http://stroke.ahajournals.org.

Cerebral Hypoperfusion Studies
Ischemia for a duration of 25 minutes was performed on the second or third postoperative day using a model similar to that previously reported. Briefly, mild fetal and maternal hypoxia was induced by administering 11% O2 air mixture to the ewe. After 10 minutes, sustained cerebral hypoperfusion was initiated by occlusion of the common brachiocephalic artery after inflation of the brachiocehalic occluder and was re-established by deflation of the brachiocephalic occluder. There were no significant differences in the physiological responses of the animals in the hypoxia-ischemia group relative to controls as summarized in Supplemental Table 1 (http://stroke.ahajournals.org).

Tissue Processing
The ewe and fetuses were euthanized (barbiturate overdose, Eutha- sol) at 1 week (control, n=8; ischemia, n=8) or 2 weeks (control, n=8; ischemia, n=8) after completion of the occlusion protocol. Heparin (1.5 mL) was administered to all fetuses via the umbilical vein. Half of the fetal brains from control or hypoxia-ischemia groups that survived for 1 or 2 weeks (n=4 per group) were immersion-fixed at 4°C in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 3 days, and then were stored in phosphate-buffered saline for at least 60 days. The remaining fetal brains were perfusion-fixed with 50 mL of 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, followed by 1% lead citrate for examination by transmission EM. Differences in the physiological responses of the animals in the hypoxia-ischemia group relative to controls as summarized in Supplemental Table 1 (http://stroke.ahajournals.org).

Ex Vivo MRI
Tissue was embedded alongside a twin control tissue block from the same level in 0.5% agarose and immersed in phosphate-buffered saline within a 4-cm diameter Plexiglas tube (custom manufactured). A custom single-turn solenoid coil (5 cm diameter, 5 cm length) was utilized for radiofrequency transmission and reception. Experiments were performed using an 11.7-T magnet interfaced with a 9-cm inner diameter magnetic field gradient coil (Bruker). Procedures generally followed the previously published strategy that used diffusion tensor imaging to characterize postmortem tissue from this and other species. Detailed scanning and image segmentation procedures are provided at http://stroke.ahajournals.org.

Immunohistochemistry
Tissue blocks were serially sectioned at 50-μm using a Leica VTS1000 vibrating microtome (Leica Microsystems). The detailed immunohistochemical protocols to visualize specific cell types were performed as previously described. Detailed immunohistochemical procedures are provided at http://stroke.ahajournals.org.

Electron Microscopy
Two 2×1×1-mm blocks were dissected from regions of apparent diffuse white matter gliosis indicated by T2 hypointensities from MRI-scanned tissue blocks and corresponding controls. Tissues were processed for electron microscopy (EM) as described. Briefly, tissues were postfixed in excess cacodylate-buffered 1% osmium tetroxide (pH 7.4), dehydrated, and embedded in epoxy resin. Cross-sections (~900-nm-thick) were stained with 1% toluidine blue and screened by bright-field microscopy. Thin sections (~90 nm) of regions of special interest were stained with 2% uranyl acetate followed by 1% lead citrate for examination by transmission EM.
Morphometric EM Studies
At least 20 images of each tissue block were randomly acquired at 56,400× magnification across the block. The total density of degenerating axons, intact axons, and axon diameter was determined by a blinded individual on a minimum of 20 randomly acquired images for each block as previously described. Briefly, an unbiased counting frame (Supplemental Figure I), which was 2 μm in width by 2 μm in length, was used for the measurement of the number of axons per 100 μm² and axon diameter. Axons were only counted when they were clearly identifiable. Axons were defined by cylindrical shape, a lack of ribosomes, and the presence of at least one microtubule. Degenerating axons were distinguished by the presence of vacuolar bodies, disrupted plasma membrane, swollen mitochondria, or dark axoplasm. Axons were counted and measured if they were partially in the counting frame and did not intersect the plane of the left side or bottom of the counting frame. The counting frame was superimposed randomly on each image (ImageJ). Axon diameter was determined by measuring the longest diameter perpendicular to the long axis of the axon to estimate the cross-sectional diameter (Supplemental Figure II).

Statistical Methods
Data analysis was performed using Prism 4 statistical software (GraphPad Software). Data were expressed as means ± 1 SEM unless otherwise noted. Comparisons were performed using analysis of variance with post hoc inference testing performed with Bonferroni multiple comparison testing. Because axon diameter data were not normally distributed, these data were analyzed with the Kruskal-Wallis nonparametric test for 1-way analysis of variance. P<0.05 was considered statistically significant.

Results
Spectrum of WMI Ranges From Small Focal Necrosis to Diffuse Non-Necrotic Lesions
To define the magnitude and distribution of axonopathy associated with hypoxia-ischemia–induced WMI, we used high-field MRI to identify early chronic lesions in glutaraldehyde-fixed brain tissue from animals that survived for 1 or 2 weeks before ultrastructural studies by EM. As recently described for paraformaldehyde-fixed brain tissue, high-field MRI similarly identified diffuse hypointense lesions in periventricular white matter that were visible on T2-weighted images in hypoxia-ischemia animals (Figure 1B) but not in controls (Figure 1A). These diffuse lesions, present in all hypoxia-ischemia animals, had fractional anisotropy and apparent diffusion coefficient values that did not differ from control (Supplemental Table II). Histopathologic analysis of diffuse lesions demonstrated that the non-necrotic lesions had increased reactive astrogliosis (glial fibrillary acidic protein) and mild microglial activation (Iba1⁺; Supplemental Figure II).

In nearly half of the animals, focal white matter lesions were identified that were hyperintense on T2 (Figure 1C) and had increased apparent diffusion coefficient (Figure 1D). Histopathologic analysis (Supplemental Figure II) demonstrated that focal MRI abnormalities corresponded to necrotic lesions defined by low levels of reactive astrogliosis (glial fibrillary acidic protein⁺) and robust microglial/macrophage activation (Iba1⁺). Hence, consistent with recent observations, the spectrum of WMI generated in this study comprised large diffuse astrogliotic lesions in all animals as well as discrete foci of necrosis in a minority of the animals.

Axon Injury Markers Identify Degenerating Axons in Focal Necrotic but Not Diffuse Non-Necrotic WMI
We next used axon injury markers to define the burden of axonopathy in necrotic foci and diffuse lesions in 1-week and 2-week survivors. Staining for neurofilament protein (SMI-312) revealed that controls (Figure 2A) and diffuse lesions (Figure 2B) had normal-appearing axons. By contrast, at both 1 and 2 weeks, lesions with macroscopic necrosis or focal microscopic necrosis (Figure 2C) displayed reduced neurofilament protein staining, axonal swellings, and spheroids consistent with axonal degeneration. Staining for β-amyloid precursor protein also visualized foci of axonal degeneration (Figure 2D). Thus, axonal degeneration was not identified in diffuse WMI but was restricted to necrotic lesions.

Ultrastructural Analysis of Axonal Degeneration in Focal Necrotic Versus Diffuse WMI
We next addressed the possibility that immunohistochemical detection of axon injury markers may be relatively insensitive...
to some forms of axonal degeneration in diffuse WMI. To analyze the ultrastructural integrity of axons, we processed for EM a superficial and deep region of periventricular white matter that corresponded to diffuse WMI identified by T2 hypointensities on MRI-scanned tissue blocks (insets, Figure 1A, 1B).

In controls (Figure 3A), degenerating axons were rarely visualized. Similarly, in lesions with diffuse WMI, rare degenerating axons were identified by the presence of vacuolar bodies, disrupted plasma membrane, and swollen mitochondria (Figure 3B). By contrast, in necrotic lesions, the number of axons was markedly reduced and degenerating axon profiles were frequently visualized (Figure 3C).

**Intact Axon Density Is Preserved in Regions of Diffuse WMI**

We next addressed the possibility that a gradual loss of axons may occur in diffuse WMI by 1 or 2 weeks after hypoxia-ischemia and would be detected by counting the total number of axonal profiles present in random fields sampled by EM (Supplemental Figure 1). In a blinded analysis of \( \approx 1000 \) intact axons per group, there were no significant changes in the density of either intact or degenerating axons between the control and hypoxia-ischemia groups at either 1 or 2 weeks in either the superficial or deep periventricular white matter (Table). In control pericallosal deep white matter, a significant increase in axon density occurred between 1 week and 2 weeks (273 \( \pm \) 347 to 410 \( \pm \) 147, \( P < 0.05 \) vs 1 wk control; analysis of variance with post hoc Bonferroni correction between groups).

**Table. Ultrastructural Analysis of Axons in Chronic White Matter Injury Found No Axonopathy in Major Lesions With Diffuse Astrogliosis at 1 Week or 2 Weeks After Hypoxia-Ischemia**

<table>
<thead>
<tr>
<th>Region</th>
<th>Axon Density (Axons/100 ( \mu m^2 ))</th>
<th>Degenerating Axon Density (Axons/100 ( \mu m^2 ))</th>
<th>Mean Axon Diameter (nm)</th>
<th>N (Axons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>Control 332 ( \pm ) 58, 9 ( \pm ) 2</td>
<td>188 ( \pm ) 14, 1163</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H-I 323 ( \pm ) 41, 11 ( \pm ) 2</td>
<td>203 ( \pm ) 15, 1256</td>
<td></td>
<td></td>
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<tr>
<td>2 wk</td>
<td>Control 344 ( \pm ) 68, 11 ( \pm ) 2</td>
<td>181 ( \pm ) 11, 958</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H-I 310 ( \pm ) 65, 12 ( \pm ) 1</td>
<td>191 ( \pm ) 10, 968</td>
<td></td>
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</tr>
<tr>
<td>DWM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>Control 273 ( \pm ) 24, 8 ( \pm ) 3</td>
<td>206 ( \pm ) 9, 925</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H-I 321 ( \pm ) 17, 11 ( \pm ) 1</td>
<td>209 ( \pm ) 13, 1362</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wk</td>
<td>Control 410 ( \pm ) 33*, 14 ( \pm ) 3</td>
<td>204 ( \pm ) 6, 1047</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H-I 364 ( \pm ) 47, 15 ( \pm ) 2</td>
<td>203 ( \pm ) 9, 993</td>
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</tbody>
</table>

*Shown are the total density of axons, density of degenerating axons, mean axon diameter, and total number of axons analyzed in each study (N). Data are presented as mean \( \pm \) SEM, n = 4 animals per group. DWM indicates deep white matter; H-I, hypoxia-ischemia; SWM, superficial white matter.

The Distribution of Axon Diameters Is Unaffected by Diffuse WMI

We next determined if a loss of axons in diffuse lesions would be detected as a change in the distribution of mean axonal diameters between the control and hypoxia-ischemia groups. There was, however, no change in the mean axonal diameter in response to hypoxia-ischemia at either 1 week or 2 weeks (Table). Figure 4 shows the distribution of axonal diameters in the regions studied at 1 and 2 weeks. Anatomic differences in the distribution of fiber diameters and peak fiber diameters were present between the superficial (Figure 4A, 4B) and deep periventricular white matter (Figure 4C, 4D), but hypoxia-ischemia did not disrupt the normal developmental distribution of axon diameters in either region at 1 week or 2 weeks after hypoxia-ischemia. Hence, diffuse WMI showed no apparent axonal loss or disruption in the normal trajectory for axon development.
Discussion

Failure of normal myelination is a major consequence of perinatal WMI, a common cause of cerebral palsy and cognitive impairment in survivors of premature birth.\(^{5,15}\) Failure to initiate myelination may be a consequence of disrupted maturation of oligodendrocyte progenitors\(^{5,4,8,16}\) or loss of axonal integrity.\(^9\) The contribution of axonal injury to myelination failure has been resistant to study for several reasons. Several markers have been studied in developing human white matter that identify degenerating axons in early necrotic lesions.\(^{4,17,18,3}\) However, the timing of WMI during premature brain development coincides with a period before the onset of myelination. Hence, it is not possible to apply markers of axonal integrity, such as nodal proteins, that are expressed later in development with the onset of myelination and are disrupted in dysmyelinated adult white matter.\(^{19,20}\) Progress to define axon integrity also has been hampered by the lack of a large preclinical animal model that closely replicates the pathophysiology and spectrum of WMI in premature infants.\(^{51}\)

We recently described a model of ovine chronic WMI in which global cerebral ischemia generates a similar spectrum and relative burden of pathology to human.\(^5\) The periventricular white matter of the preterm fetal sheep has few myelin-ated axons, consistent with its immature state. With this model, ex vivo high-field MRI identified novel signal abnormalities that histologically correspond to microscopic necrosis and diffuse non-necrotic WMI.\(^5\) These lesions have been previously undetected at lower field strength.\(^{22}\) We found striking differences in the burden of axonal degeneration in necrotic and diffuse WMI defined by a combination of light microscopy and transmission EM. Quantitative EM studies, guided by high-field MRI, have not been applied before to study axon integrity in perinatal WMI. This approach allowed us to visualize both overt and subtle changes in the integrity of developing axons that are not feasible to detect by light microscopy. The most significant axonal degeneration was visualized in necrotic lesions that were enriched in reactive microglia and depleted in astrocytes. Lesions were confirmed to be necrotic by the presence of dystrophic axons and axonal spheroids, which degenerate during the early phase of coagulative necrosis.\(^{23–26}\) Reduced myelination secondary to neuro-axonal degeneration occurs in perinatal rodents after hypoxia-ischemia\(^{27,28}\) and often manifests as significant cerebral atrophy, as occurs in cystic necrotic human lesions.\(^{18,29}\) By contrast, cortical neuronal loss is uncommon when only diffuse astrogliotic lesions occur in human WMI.\(^{29}\)
sheep, the burden of microscopic necrosis is very low. Here, we found that most axonal degeneration was restricted to these small necrotic foci. The clinical significance of these lesions is unclear given their small size, but it is likely to be influenced by the location of the lesion.

During development, small premyelinated axons are particularly susceptible to glutamate-mediated excitotoxicity at sites of contact with oligodendrocyte processes that involves both N-methyl-D-aspartic acid receptors and N-methyl-D-aspartic acid glutamate receptors. Glutamate-mediated axonal injury is related to excessive glutamate depletion from oligodendrocytes and axons, which appear to be the major sources of extracellular glutamate during energy failure from hypoxia-ischemia. Thus, a potential approach to prevent myelination failure in necrotic lesions may be to couple cerebral hyperthermia with agents that block axonal degeneration via pathways that mediate excitotoxic injury.

The paucity of axonopathy in diffuse WMI is consistent with a mechanism of myelination failure that differs substantially from necrotic WMI. Axonal degeneration was not detected in early non-necrotic diffuse WMI in human or fetal sheep. Axonal degeneration was observed in diffuse WMI adjacent to large acute or organizing necrotic lesions, because axons traversed across necrotic foci to adjacent non-necrotic lesions. Large necrotic lesions are now clinically uncommon and our data support that significant axonopathy does not occur in diffuse WMI in the absence of large necrotic lesions. Although we found no structural evidence of abnormal axons, we cannot exclude that reactive glia generate a chronic inflammatory response that results in axonal dysfunction. Electrophysiological studies would be needed to address this possibility.

An alternative mechanism for myelination failure in diffuse WMI involves disturbances in OL lineage maturation. Preterm neonates are susceptible to acute diffuse WMI triggered by selective vulnerability of pre-OL, as opposed to other glia, neurons, or axons. Acute degeneration of pre-OL triggers reactive astrogliosis and a compensatory increase in pre-OL that fail to differentiate to mature OL. Thus, pre-OL maturation arrest within diffuse WMI may contribute to chronic myelination failure in preterm survivors.

Conclusions
In summary, our findings support that axonal degeneration occurs in association with discrete foci of microscopic necrosis with pan-cellular loss. Axonopathy was not detected within diffuse WMI, where astroglisis and pre-OL maturation arrest were previously observed. Hence, the primary mechanism of myelination failure in diffuse WMI appears to involve an aberrant reaction to injury in which pre-OLs mount a disrupted repair response with arrested maturation. Therapies directed at prevention of axonal degeneration would optimally target mechanisms related to necrotic WMI, whereas in diffuse lesions, where axons are present, alternative strategies will be required that promote arrested pre-OL maturation. Within adult demyelinating lesions, hyaluronan triggers the arrest of pre-OL maturation. One strategy for the preterm neonate may be to target the accumulation of hyaluronan within the extracellular matrix of diffuse lesions.

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Disclosures
None.

References


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Differential susceptibility to axonopathy in necrotic and non-necrotic perinatal white matter injury

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From the Departments of Pediatrics¹, Behavioral Neuroscience², the Advanced Imaging Research Center³, the Oregon National Primate Research Center⁴, Obstetrics and Gynecology⁵, and Neurology⁶ Oregon Health & Science University, Portland, Oregon.
Supplemental Materials and Methods

Animal Surgery
Ewes with twin pregnancies were studied so that each experimental animal had a twin control. The ewe was initially anesthetized with intravenous ketamine (5 mg/kg) and diazepam (0.13 mg/kg), an endotracheal tube placed, and anesthesia maintained with 1% halothane in O₂ and N₂O. Maternal end tidal PCO₂ and oxygen saturation were monitored continuously. A midline laparotomy and a hysterotomy were performed in a sterile field and the fetus exposed. Non-occlusive vinyl catheters were placed in a carotid artery. The vertebral-occipital arteries were isolated bilaterally and ligated with silk suture. In sheep, these anastomoses connect the vertebral arteries supplied by the thoracic aorta with the external carotid arteries that are fed by the common brachiocephalic.¹ A hydraulic occluder (silastic) was placed on the common brachiocephalic artery. Finally, an amniotic fluid catheter was placed and one million units of penicillin G were given when the uterus was closed.

Physiological Monitoring
On the second or third post-operative day, at least 30 minutes prior to the start of the experiment, pressure transducers and a chart recorder (PowerLab 16/30, ADInstruments, Sydney, Australia) recorded pressure in the fetal artery relative to amniotic fluid (MABP). Fetal heart rate (HR) was calculated from triplicate measurements of the arterial pressure pulse intervals over a continuous recording of no less than 20 seconds.

Blood analysis
Blood samples (1 mL) taken anaerobically from the fetal carotid artery were analyzed for arterial pH, P₂O₂, P₂CO₂ corrected to 39°C, hemoglobin content (Thb), arterial oxygen saturation (SatO₂) and hematocrit (Hct), glucose (Glu), lactate (Lac) and arterial oxygen content (CaO₂; ABL725 blood gas analyzer, Radiometer Medical A/S, Bronshoj, Denmark). After a 72 h recovery from surgery, fetuses were entered into the study if they demonstrated normal fetal oxygenation, defined as > 6 ml O₂/100 ml blood.

Ex vivo Magnetic Resonance Imaging
A Stejskal-Tanner multi- slice spin-echo pulse sequence (δ = 12 ms, Δ = 21 ms, and G = 11.6 G/cm; resulting in b = 2.5 μs/mm²) was used for DTI measurements. The b-value for this study was selected to provide an approximate match in diffusion sensitization to a typical in vivo measurement in which b = 1 μs/mm² (the water apparent diffusion coefficient is ~2.5-fold smaller in post-mortem tissue than in vivo).² Diffusion anisotropy measurements were made using a 25-direction, icosahedral sampling scheme ³ in combination with two measurements in which b = 0. Other pulse sequence settings were TR = 10 s, TE = 42 ms and NEX (the number of averaged transients) = 1. The image resolution was isotropic, with voxel dimensions of 0.3 mm and a 128 voxel (phase-encode) by 256 voxel (readout) by 70 voxel (slice-select) field of view. Standard procedures³ were followed to determine eigenvalues (λ₁, λ₂, and λ₃, listed from smallest to largest) and the signal amplitude in the absence of diffusion weighting for each voxel from the set of 27 3D images. The apparent diffusion coefficient (ADC = (λ₁ + λ₂ + λ₃)/3) and fractional anisotropy (FA, defined in ⁴) were calculated from the eigenvalues for each voxel.

Three sets of T₂-weighted (T₂w) images were acquired in the same session with pulse sequence settings of TR = 10 s, TE = 40, 80 and 120 ms and NEX = 3, and image
resolution was isotropic with voxel dimensions as above. T2 maps were generated by fitting T2 as a function of signal decay.\(^5\)

**MRI segmentation.**

Voxels for each tissue block from the 2 regions analyzed for electron microscopy were manually classified by an individual who was blinded to neuropathologic classification using the derived T\(_{2w}\) image, ADC map, and standard functionalities of the ITK-snap program (http://www.itksnap.org).\(^6\) Mean values for MRI-derived parameters within WM reported herein for a given case were computed as means over the set of voxels classified as WM.

**Immunohistochemistry**

Glutaraldehyde fixed tissue sections underwent antigen retrieval in 50 mM Citrate buffer (pH 6.0) for 10 min at 90 °C prior to immunostaining. No antigen retrieval was required in paraformaldehyde-fixed tissue. Astroglia were visualized with rabbit glial fibrillary acidic protein (GFAP) antisera (1:500, Z-0334; DAKO, Carpinteria, CA). Microglia and macrophages were visualized with a rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody (1:500; 019-19741; Wako Chemicals, Richmond, VA). Axons were visualized with the pan-axonal neurofilament marker, mouse monoclonal antibody SMI-312 (1:1000; SMI-312R; Covance, Berkley, CA). Appropriate anti-mouse and anti-rabbit AlexaFluor secondary antibodies were used for visualization of primary antibodies (1:500; Invitrogen, Carlsbad, CA) in paraformaldehyde-fixed tissue. In gluteraldehyde-fixed tissue, secondary antibodies were visualized using the peroxidase-immunoperoxidase reaction (Vectastain ABC Kit; PK4000; Vector Laboratories, Burlingame, CA) and 3,3’-diaminobenzidine with nickel-enhancement (SK4100; Vector Laboratories). For fluorescent immunohistochemical studies, tissue sections were counterstained with Hoechst 33342 (Invitrogen).
Supplemental Table S1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>H-I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BP</strong></td>
<td>Basal</td>
<td>32.5 ± 2.6</td>
</tr>
<tr>
<td>(mmHg)</td>
<td>Hypoxia</td>
<td>29.1 ± 3.7 *</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>35.5 ± 3.0</td>
</tr>
<tr>
<td><strong>HR</strong></td>
<td>Basal</td>
<td>202 ± 15</td>
</tr>
<tr>
<td>(beats/min)</td>
<td>Hypoxia</td>
<td>218 ± 16</td>
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<tr>
<td></td>
<td>Recovery</td>
<td>206 ± 16</td>
</tr>
<tr>
<td><strong>pHa</strong></td>
<td>Basal</td>
<td>7.37 ± 0.01</td>
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<td>Hypoxia</td>
<td>7.40 ± 0.01 **</td>
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<td></td>
<td>Recovery</td>
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<td>Basal</td>
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<td>(mmHg)</td>
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<td>(mmHg)</td>
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<td></td>
<td>Recovery</td>
<td>24.8 ± 3.8</td>
</tr>
<tr>
<td><strong>Hb</strong></td>
<td>Basal</td>
<td>9.29 ± 0.85</td>
</tr>
<tr>
<td>(g/dL)</td>
<td>Hypoxia</td>
<td>9.41 ± 0.97</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>9.47 ± 0.78</td>
</tr>
<tr>
<td><strong>SatO₂</strong></td>
<td>Basal</td>
<td>67.5 ± 5.2</td>
</tr>
<tr>
<td>(%)</td>
<td>Hypoxia</td>
<td>27.4 ± 5.4 **</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>65.0 ± 9.7</td>
</tr>
<tr>
<td><strong>Hct</strong></td>
<td>Basal</td>
<td>28.8 ± 2.3</td>
</tr>
<tr>
<td>(%)</td>
<td>Hypoxia</td>
<td>29.1 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>29.3 ± 2.3</td>
</tr>
<tr>
<td><strong>Glu</strong></td>
<td>Basal</td>
<td>1.28 ± 0.34</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>Hypoxia</td>
<td>1.24 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>1.52 ± 0.42</td>
</tr>
<tr>
<td><strong>Lac</strong></td>
<td>Basal</td>
<td>1.07 ± 0.12</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td>Hypoxia</td>
<td>1.43 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>3.23 ± 1.10 **</td>
</tr>
<tr>
<td><strong>CaO₂</strong></td>
<td>Basal</td>
<td>8.06 ± 0.67</td>
</tr>
<tr>
<td>(Vol%)</td>
<td>Hypoxia</td>
<td>3.23 ± 0.90 **</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>8.23 ± 1.05</td>
</tr>
</tbody>
</table>

**Summary of sheep physiological responses to hypoxia-ischemia (H-I).**

Data presented as mean ± standard deviation. The data comprise the mean physiological responses for animals subjected to H-I that survived for 1 or 2 weeks. Comparisons were made between basal and hypoxia and recovery control values and between corresponding control and H-I values. *, p < 0.05 vs. basal control; **, p < 0.001 vs. basal control; †, p < 0.05 vs. control; ††, p < 0.001 vs. control. All statistical analysis employed ANOVA with post hoc Bonferroni between groups.
### Supplemental Table S2

<table>
<thead>
<tr>
<th>Region</th>
<th>T$_2$ (ms)</th>
<th>ADC (mm$^2$/ms$^2$)</th>
<th>FA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SWM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>144 ± 11</td>
<td>0.56 ± 0.05</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>H-I</td>
<td>96 ± 13 *</td>
<td>0.67 ± 0.07</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>2 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>98 ± 12 †</td>
<td>0.66 ± 0.02</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>H-I</td>
<td>85 ± 9</td>
<td>0.71 ± 0.05</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td><strong>DWM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>119 ± 7</td>
<td>0.53 ± 0.06</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>H-I</td>
<td>81 ± 6 *</td>
<td>0.60 ± 0.07</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>2 wk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>84 ± 14</td>
<td>0.64 ± 0.02</td>
<td>0.23 ± 0.01 †</td>
</tr>
<tr>
<td>H-I</td>
<td>77 ± 9</td>
<td>0.67 ± 0.05</td>
<td>0.19 ± 0.01</td>
</tr>
</tbody>
</table>

**Summary of the analysis of MRI signal abnormalities (T$_2$, ADC and FA) for the regions of interest (ROIs) where EM blocks were analyzed.** As shown in Figure 1, diffuse hypo-intense signal abnormalities on T$_2$ images identified periventricular white matter lesions from within which the tissue blocks were selected for the ultra-structural analysis of axons by electron microscopy (EM). Data are presented as mean ± SEM, n = 4 animals per group. Note that significant differences in T$_2$ were observed for the 1 and 2 week survivors in the superficial periventricular white matter (SWM) or deep periventricular white matter (DWM), as indicated: *, p < 0.05 vs. 1 week (wk) control; †, p < 0.05 vs. 2 week control. Between 1 and 2 weeks, controls displayed a significant normal developmental increase in FA: †, p < 0.05. All statistical analysis employed ANOVA with post hoc Bonferroni between groups.
Supplemental Figures and Figure Legends

Supplemental Figure S1

Stereological assessment of axonal caliber and density. A and B. Example measurements of intact axons with an unbiased counting frame. A 4 mm² counting frame was randomly superimposed on electron micrographs. The lines within numbered axons represent the axon diameter as measured. Obliquely cut axon diameters were measured perpendicular to the long axis of the axon (arrows). Scale bars: 667 nm.
Supplemental Figure S2

Spectrum of injury assessed by GFAP and Iba1. A and B, Typical astrocyte (GFAP) and microglia/macrophage (Iba1) staining patterns in control WM, respectively. C, Marked astrogliosis was observed in regions of diffuse white matter injury. D, Ramified microglia (box and inset) were present in these regions of diffuse white matter injury. E, Macroscopic necrosis could be identified by regions of ameboid microglial infiltration. F, Microscopic necrosis were visualized by small focal regions of ameboid microglial infiltration (box and inset) with clear borders (arrows). Scale bars: 40 mm. Inset: 30 x 30 mm.
Supplemental References


