Published in final edited form as:

Ann Neurol. 2014 April ; 75(4): 533–541. doi:10.1002/ana.24115.

Unmyelinated Axon Loss with Postnatal Hypertonia after Fetal Hypoxia

Alexander Drobyshvsky, PhD¹, Rugang Jiang, PhD¹, Laixiang Lin, PhD¹, Matthew Derrick, MD¹, Kehuan Luo, MD¹, Stephen A. Back, MD, PhD², and Sidhartha Tan, MD¹

¹From the Department of Pediatrics, NorthShore University HealthSystem Research Institute, Evanston, IL

²Department of Pediatrics, Oregon Health Science University, Portland, OR

Abstract

Objective—White matter (WM) injury due to myelination defects is believed to be responsible for the motor deficits seen in cerebral palsy. We tested the hypothesis that the predominant injury is to functional electrical connectivity in unmyelinated WM fibers by conducting a longitudinal study of central WM tracts in newborn rabbit kits with hypertonia in our model of cerebral palsy.

Methods—Pregnant rabbits at 70% gestation underwent 40-minute uterine ischemia. Motor deficits in newborn kits, including muscle hypertonia, were assessed by neurobehavioral testing. Major central WM tracts, including internal capsule, corpus callosum, anterior commissure, and fimbria hippocampi, were investigated for structural and functional injury using diffusion tensor magnetic resonance imaging (MRI), electrophysiological recordings of fiber conductivity in perfused brain slices, electron microscopy, and immunohistochemistry of oligodendrocyte lineage.

Results—Motor deficits were observed on postnatal day 1 (P1) when WM tracts were unmyelinated. Myelination occurred later and was obvious by P18. Hypertonia was associated with microstructural WM injury and unmyelinated axon loss at P1, diagnosed by diffusion tensor MRI and electron microscopy. Axonal conductivity from electrophysiological recordings in hypertonic P18 kits decreased only in unmyelinated fibers, despite a loss in both myelinated and unmyelinated axons.

Interpretation—Motor deficits in cerebral palsy were associated with loss of unmyelinated WM tracts. The contribution of injury to myelinated fibers that was observed at P18 is probably a secondary etiological factor in the motor and sensory deficits in the rabbit model of cerebral palsy.

Cerebral palsy (CP) is the most common cause of severe physical disability in childhood,¹ resulting from an injury to the developing central nervous system, particularly in white matter (WM).² Hypoxia–ischemia (H-I) is a major pathological mechanism for CP.³ The characteristic signs CP are hypertonia, movement disorders, muscle weakness, ataxia, and rigidity.⁴ Hypoxic–ischemic encephalopathy in term babies results in structural injury, seen

Address correspondence to Dr Drobyshvsky, Department of Pediatrics, NorthShore University HealthSystem Research Institute, 2650 Ridge Ave, Evanston, IL 60201. oldrobys@gmail.com.

Potential Conflicts of Interest

Nothing to report.

in the first week, to the posterior limb of the internal capsule (IC) by apparent diffusion coefficient by magnetic resonance imaging (MRI), and the extent of structural injury is correlated with a severe neuromotor outcome.⁵ Hemiparesis following periventricular leukomalacia is associated with decreased fractional anisotropy (FA) of the corticospinal tract, suggesting injury to fiber tract integrity, at 12 to 16 years.⁶

Hypertonia is a pathognomonic feature of CP, but little is known about how it develops. A possible explanation for muscle hypertonia is decreased functional input from the descending corticospinal tract.^{7,8} Structural evidence of injury to corticospinal tract is accompanied by decreased supraspinal control⁹ leading to clinical hypertonia, as well as a congenital absence of corticospinal tracts.¹⁰ Myelination deficits are thought to be a major etiological factor in development of CP in premature infants,¹¹ but severe cases of CP present with clinical hypertonia soon after birth,¹² when most of the WM tracts are unmyelinated.¹³ Most CP patients survive, so myelination deficits can only be evaluated noninvasively, and not histologically.¹⁴ Animal models could elucidate the pathophysiology of motor deficits in CP, but there has been a lack of appropriate models recapitulating the most essential feature of human CP—hypertonia and postural deficits.¹⁵ In rodent and primate models, the motor deficits are mild.^{16–18} In contrast, the antenatal global H-I rabbit model exhibits pronounced motor abnormalities, including muscle hypertonia.^{19,20} In this model, there is a combination of cortical and deep gray matter neuronal injury²¹ and WM injury, which correlates with abnormal motor outcome.²²

Two large knowledge gaps are how WM fiber tracts function in hypertonia and how the different developmental trajectories in different WM tracts¹³ influence structural and functional injury in those tracts after antenatal H-I. There are few studies on the functional properties of central WM fiber tracts, particularly during perinatal development. We previously showed that the perinatal functional development trajectories of the electrophysiological compound action potential (CAP) correlated with MRI estimations of structural WM development.²³ As myelination deficits in the posterior limb of the IC are associated with CP,¹⁴ our hypothesis was that disruption of functional corticospinal connectivity in the IC could explain the manifestations of hypertonia via mechanisms involving either axonal injury, disruption of myelination, abnormal conductivity in unmyelinated and myelinated fibers, or total axonal loss. Structural injury to axons was assessed in the same regions of interest (ROIs) on diffusion tensor imaging (DTI) and by electron microscopy (EM) and compared to functional electrophysiological changes, characterized by conduction velocity (CV) and CAP in developing newborn rabbit kits.

If preoligodendrocyte (preOL) injury is the only or central pathological target leading to CP, then one would expect to see differential windows of vulnerability to injury corresponding to the developmental trajectories of oligodendrocyte (OL) maturation and myelination. To test this hypothesis, we compared 4 different central WM fiber tracts with different myelination trajectories.¹³

Materials and Methods

All animal procedures were approved by the institutional animal care and use committee of NorthShore University HealthSystem.

Animal Model of Global Fetal Hypoxia and General Plan of Experiments

New Zealand White pregnant rabbits at 22 days gestation (70% term) underwent uterine ischemia induced by inflation of an intra-aortic balloon catheter, which caused in vivo global fetal H-I.¹⁹ Surviving rabbit kits were allowed to grow until postnatal day 18 (P18). At P1, P11, and P18, the kits underwent neurobehavioral testing to determine the extent of motor and sensory deficits, including muscle tone measurements,¹⁹ and were divided into hypertonic, nonhypertonic, and control groups. Control kits came from unmanipulated litters.

MRI Methods

Serial in vivo DTI experiments were conducted on a 9.4T Bruker magnet with $b = 0$ for 0.8 ms/ μm^2 . The number of kits was 11 and 21 for P1, 8 and 6 for P11, 8 and 7 for P18, for the control and hypertonia groups, respectively. Apparent diffusion coefficient, directional diffusivities, and FA maps were calculated. T2 relaxation time maps were obtained using multiecho sequence, followed by fitting the signal decay curve in the T2-weighted images with different echo times. ROIs on IC, fimbria hippocampi (FH), anterior commissure (AC), and corpus callosum (CC) were outlined.

Electrophysiology Methods

The brain of rabbit's kits (7 and 13 at P1, 5 and 4 at P11, 8 and 6 at P18, for the control and hypertonia groups, respectively) was sectioned into 400 μm slices containing CC, FH, IC, and AC, and perfused with oxygenated artificial cerebrospinal fluid. CAP was measured at 4 fixed distances along a line between stimulating and recording electrodes,²³ starting at the farthest distance. CV and CAP latency intercept were derived from linear regression of CAP response latency at various recording distances of waveforms that satisfied recording from a single fiber (shorter onset, increasing amplitude). CAP area was calculated by integration area under the negative peaks of the response curve. Recordings were performed in both the left and right IC and FH and also averaged. Both sides were recorded because a laterality effect has been reported in H-I injury²⁴ and because we have occasionally observed hypertonia and postural changes restricted to one side. Number of response peaks in the recorded CAP of all WM fibers varied from 1 to 3. To characterize properties of unmyelinated and myelinated fibers in central WM, fast and slow response peaks in each fiber tract were analyzed separately. In the recordings where triple peaks were present, the peaks with the slowest CV were considered representative of unmyelinated fiber tracts and the peaks with the fastest CV of myelinated fiber tracts. The 99th percentile of CV values of the unmyelinated and myelinated fiber responses was used to assign predominantly unmyelinated or myelinated status to single or double peak recordings. Intermediate CV values were considered to belong to mixed fiber tracts, containing unmyelinated and partially myelinated fibers, and were reported separately.

EM

Sections were cut perpendicular to the fiber course, corresponding to the position between electrophysiological electrodes in IC in P18 kits (n = 6 controls and n = 7 hypertonic after H-I), processed for EM, and imaged on a JEOL (Tokyo, Japan) 1230 Transmission Electron Microscope. Density of myelinated and unmyelinated axons, proportion of myelinated fibers, and myelinated area were quantified using stereological methods in a blinded fashion.

Quantification of OL lineage stages

Serial sections were obtained in IC (n = 10 controls and n = 8 hypertonic after H-I) and AC (n = 5 controls and n = 5 hypertonic after H-I) WM tracts of P1 kits. The detailed immunohistochemistry protocols to visualize specific stages in the OL lineage were as described earlier.²⁵ Total OL cell density, cell density, and fractions of preOLs, immature OLs, and mature OLs were estimated in a blinded fashion.

Results

Hypertonia in P1 Kits Is Marked by Microstructural WM Abnormalities and Loss of Unmyelinated Axons

Newborn rabbit kits at P1, 10 days after fetal H-I injury, exhibited an array of motor abnormalities (Fig 1A, insert), as reported previously.¹⁹ Most prominent was the finding of hypertonia at P1. The evolution of tone from hypotonia to hypertonia after H-I at embryonic day 22 (E22) progresses rapidly in rabbits²⁶ and probably occurred in utero. At P1, rabbit WM was unmyelinated (Fig 1). Despite the lack of myelination, it had high FA on DTI imaging, enabling the outlining of major WM fiber tracts, including AC, FH, CC, and IC. This could be explained by a larger restriction of water movement across than along the fiber course. In hypertonic P1 kits, FA significantly decreased in the IC, caused by an increase in radial diffusivity and not axial diffusivity. A composite index of fiber tract integrity, the product of FA and tract cross-sectional area (FAxArea), was decreased in most fiber tracts. The cross-sectional area, as an index of tract volume, was not significantly decreased in the hypertonic group at P1, except for the CC. T2 relaxation time was unchanged at P1. No significant changes in MRI indices were found in the H-I nonhypertonic group.

Hypertonic Kits Had No Change in OL Numbers in the IC or AC

We performed a count of OL lineage at P1 only because later counts were unreliable due to the appearance of myelin. No significant differences were found between hypertonia and control P1 groups in density of preOL, immature OL, total OL, or fractions of each OL subpopulation (Fig 2A, B). Fraction of mature OL was low at this age (<1%) and was not different between groups.

Hypertonic Kits Have Unmyelinated Axonal Loss

Microstructural WM abnormalities on MRI corresponded to the loss of unmyelinated axons in hypertonic P1 kits on electron microphotographs and confirmed by stereological count of axons (see Fig 2C, D; $p = 0.0011$). Total axon number in the IC was significantly lower in hypertonic kits, despite the maintenance of the tract volume. Comparison of the axon size

distribution (see Fig 2E) revealed that smaller axons ($<0.25\mu\text{m}$) were lost preferentially (chi-square, $p = 0.025$).

Conduction of Surviving Unmyelinated WM Is Preserved at P1

Electrophysiological properties of CAP in central WM tracts were not different between hypertonic H-I, nonhypertonic H-I, and control groups at P1, such as CV and CAP area (see Fig 2G, H). CAP area was significantly larger in CC in hypertonic kits at P1 (2.97 ± 0.17 vs $1.68\pm0.16\text{mV/ms}$ in controls), and there was a smaller CAP latency intercept in the IC at P11 (1.12 ± 0.10 vs 1.50 ± 0.07 milliseconds in controls).

Loss of Unmyelinated and Myelinated Fibers in IC in Hypertonic P18 Kits

At P18, FA and directional diffusivities were not different between hypertonic and control kits in the majority of the WM tracts (Fig 3A, B), but the composite index, F_{Ax}Area, was decreased in hypertonic kits in all WM tracts (see Fig 3C). The cross-sectional area was significantly lower in hypertonic kits at P11 and P18. T2 relaxation time in hypertonic kits was also significantly higher than in controls at P18, possibly reflecting less myelination.

Axonal density and axonal number in IC in hypertonic kits were determined by EM (see Fig 3). The total myelinated axon count (the product of EM axonal density by MRI cross-sectional area) was significantly lower in hypertonic kits at P18, as was the total number of unmyelinated axons. The loss in axon numbers was predominantly due to the decrease in the tract crosssectional area in hypertonic kits, as the density of unmyelinated and myelinated axons was not different. There were no differences in the mean diameter of myelinated or unmyelinated axons, the cross-sectional area occupied by myelinated axons, or the axonal size distribution (Fig 4).

Functional Deficits in Unmyelinated Fibers of Hypertonic P18 Kits

We reasoned that complete axonal loss would result in no conduction. We therefore recorded the number of attempts to obtain a CAP signal (the numbers in the bars in Fig 5). The number of successful recordings of both unmyelinated and myelinated tracts was decreased in hypertonic kits. The average of right and left recordings is depicted for clarity in Figure 5. When recorded, the CV significantly decreased in unmyelinated fibers of the right IC (but not the left IC; data not shown for clarity), and in the averaged left and right IC recordings decreased from 292.5 ± 8.1 to $259.2\pm13.0\text{mm/s}$, indicating that the conduction of unmyelinated fibers may be an important factor in the motor deficits. Interestingly, there was a larger CAP area in unmyelinated fibers in the hypertonic group, indicating a possible compensatory response. When a myelinated response was obtained, no differences in electrophysiological properties were found.

Discussion

The most consistent finding of the study was the loss of fibers in newborn hypertonic kits after antenatal H-I, based on DTI parameters (F_{Ax}Area) and confirmed by axonal count on EM at P18. The functional data (CAP recordings) did not support axon dysfunction at P1 (no change in any CAP parameters), and the changes at P18 were complex and inconsistent

(increased CV in unmyelinated CC, decreased in IC). No changes in OL lineage and no difference in functional properties were found in myelinated fibers in hypertonic kits. This study is the first to emphasize the relative importance of injury to unmyelinated WM fibers, particularly in the IC, in the pathogenesis of hypertonia and motor deficits following antenatal H-I. Decrease in number and less functional unmyelinated fibers were associated with hypertonia. The contribution of myelinated fiber loss that was observed at P18 is probably a secondary factor in the motor deficits in the rabbit model of cerebral palsy.

Unmyelinated Axon Loss and Origin of Motor Deficits

In humans, myelination injury has been implicated in the manifestations of CP. CP is typically diagnosed at 18 to 24 months, coinciding with advanced myelination of corticospinal tracts.²⁷ In the current study, injury to unmyelinated WM was associated with the manifestations of motor deficits and hypertonia following fetal H-I in rabbits, as early as P1, when central WM was unmyelinated. Microstructural abnormalities detected by DTI in hypertonic P1 kits reflected injury to unmyelinated fibers, and that was confirmed by significant unmyelinated fiber loss in IC on EM.

Currently, the origin of hypertonia in CP is attributed to a decreased functional input of descending corticospinal tracts and disinhibition of spinal motoneurons.⁷ This may result from a decrease in conduction in partially myelinated descending fibers and/or a decrease in number of axons. Loss of motor cortical axonal projections to spinal cord after H-I in P9 mice was implicated in the development of later subtle motor deficits.²⁸ In our rabbit model, despite structural injury to WM, we found no overt loss in function of surviving unmyelinated axons in the hypertonic group at P1 and P11. CC actually had higher CAP area at P1 and higher CVs at P18, whereas IC had smaller CAP latency intercept at P11 and larger CAP area in unmyelinated fibers at P18 in hypertonic kits (see Figs 2, 5), indicative of a possible loss in smaller size axons after injury or that the surviving axons had a compensatory plastic response. By P18, functional deficits of the unmyelinated axons were detected as evidenced by significantly smaller CV in IC (see Fig 5A).

Unmyelinated WM Injury after Antenatal H-I

Structural WM injury in rabbits was characterized by decreased FA and cross-sectional area of tracts, and increased radial diffusivity at P1 and P11, consistent with our findings in humans.²⁹ In P1 hypertonic kits, there was significant axonal loss in the IC on EM and decreased WM tract volumes by MRI. Interestingly, DTI revealed structural abnormalities but little change in the WM tract volumes. In contrast, at P18 there was a larger decrease in tract volume with fewer DTI abnormalities, possibly due to tract shrinkage and elimination of excessive extra-axonal space. We introduced the product of FA and cross-sectional WM tract area as a clinical biomarker of fiber integrity²⁹ in humans. In rabbits, this diagnosed injury at both P1 (see Fig 1F) and at P18 (see Fig 3C).

Cortical and subcortical neuronal injury was found 24 hours after H-I,²¹ implying that the predominant mechanism of injury in rabbits is probably secondary to degeneration from gray matter injury. This injury is seen in more severely affected human infants with mixed CP and not typically preterm survivors with spastic diplegia without oromotor feeding

difficulties and upper extremity spastic dystonia. Preterm brain injury resulting in hypomyelination from OL injury^{30–32} is considered to be a major pathophysiological event of CP.^{33–37} However, it is being increasingly recognized that gray matter injury commonly occurs along with WM injury.^{38–41}

In other models of fetal brain injury, such as the fetal sheep 4-vessel occlusion model, there is a relative preservation of neuroaxonal elements.⁴² Axonal loss is found in sites of focal necrosis, but not in areas of astrogliosis, defined as diffuse WM injury by hypointensity on T2-weighted MRI. In rabbits, we did not observe diffuse T2 hypointensity lesions in WM. The non-necrotic diffuse WM injury and lack of axonopathy in the preterm sheep could be attributed to the earlier onset of myelination and relative abundance of WM compared to rabbits. Axonal loss in IC was found specifically in hypertonic rabbit kits as opposed to all survivors of H-I. This is an important distinction, as previous perinatal models of H-I were not able to elucidate pathophysiological mechanisms underlying motor deficits of CP, because the models investigated H-I and not specifically H-I with motor deficits.

Unmyelinated Axon Injury, Maturation Arrest of Oligodendroglia, and Hypomyelination

Hypomyelination in periventricular leukomalacia has been thought to involve primary injury to preOLs in the WM, resulting in a subsequent dysfunction of myelinating OLs.⁴³ Signs of hypomyelination in IC in hypertonic rabbit kits at P18 included increased T2 relaxation time, decreased density of axons, decreased myelinated area, and decreased EM count in myelinated axons (see Fig 3). The number of successful myelinated fiber recordings also decreased (see Fig 5E). preOLs are considered to be selectively vulnerable to H-I injury.³⁰ However, we did not find significant differences in preOLs, immature OLs, or mature OLs between hypertonia and control groups at P1. preOLs have been reported to proliferate increasingly after initial depletion in response to H-I injury,⁴⁴ which was also observed in rabbits after fetal H-I.²¹ At E22, preOLs have not reached their peak, and H-I at this stage could result in less OL injury. The presence of sufficient numbers of OLs in WM injury suggests maturation arrest as a contributory factor in hypomyelination at P18.⁴⁴ The maturation arrest could also include dysfunction of the OL–neuron unit, which may contribute to the functional deficit of the unmyelinated axons.

Motor deficits evolve with age, and later myelination deficits could worsen injury. The converse could also be true, as myelination mitigates the initial unmyelinated injury. We find a slight improvement in tone in the severe ranges (albeit not enough to make the kits satisfy the criteria of normal tone), a finding also observed in some humans.

Susceptibility of Different Fiber Tracts to H-I Injury

The period of peak vulnerability of WM is thought to be when axons undergo radial expansion during the initiation of early myelination.⁴⁵ H-I injury at E22 occurs before myelination and even before the period of preOL proliferation. Due to the different timing in maturation in central WM tracts,¹³ an idea of vulnerable periods affecting axons could be obtained by comparing injury to different WM tracts. We observed significant FA decrease after H-I injury in all studied fiber tracts at P11 and in CC in all studied ages. Areas of AC and IC were significantly less in P18 in the hypertonic group. With the exception of IC, no

differences in AC, CC, or FH were found between hypertonia and control groups in radial diffusivity, cross-section area, T2 time, CV, or CAP area at either P1 or P18. However, structural injury to all fiber tracts was found using Fx-Area index with similar magnitude of changes between the tracts. Taken together, there is not enough evidence to suggest that the injury to IC after antenatal H-I at E22 was greater than that to FH (which develops earlier than IC) or AC and CC (which develop later than IC). A possible explanation could be the timing of injury, the differences in OL development between the tracts not being distinctive enough at E22.²¹

Alix and Fern have shown small > large premyelinated axonal injury in the optic nerve following oxygen– glucose deprivation (OGD) ex vivo in P8 to P12 rodents.⁴⁶ Additionally, they have shown that large pre-myelinated axons are vulnerable to OGD,⁴⁵ with injury observed on EM, which was implicated as a possible mechanism of myelination failure. CV was not measured in this study. In contrast, we utilized the differences in CV between unmyelinated and myelinated axons to detect possible myelination deficits. CV of myelinated and mixed tracts were not different at P18 between hypertonic and control animals, however. No selective vulnerability of large unmyelinated fibers was observed in rabbit WM, possibly due to different timing of OL maturation and myelination between rats and rabbits. Rabbits are unlike rodents in that they are perinatal brain developers, similar to humans; rodents are postnatal.⁴⁷

Although we observed histological evidence of myelination in CC at P18 and recorded dual peaks in CAP responses, CV of the fast component did not satisfy our criteria of myelinated fiber (99th percentile limits of myelinated and unmyelinated fibers). Therefore, we assigned responses in CC to “mixed” fibers, which may include both types. Despite a decrease of FA and a nonsignificant decrease in FxArea, CV in unmyelinated fibers in CC was higher in hypertonic kits. This observation of apparent plastic response suggests that future studies should incorporate functional and structural studies to obtain a more comprehensive picture of perinatal WM injury.

Concluding Remarks

Unmyelinated WM fibers are important in antenatal H-I injury, particularly in the IC, in the pathogenesis of hypertonia and motor deficits, more so than myelinated fibers. Hypertonia is strongly associated with a decrease in the number of unmyelinated fibers, less with axonal dysfunction.

Acknowledgments

Supported by the NIH (NINDS NS43285, NINDS NS051402, S.T.; NICHD HD057307, M.D.; NINDS NS045737, S.A.B.) and the UCP/Hearst Foundation, A.D.), 1 S10 RR15685-01.

We thank X. Ji and A. Liu for technical help.

References

1. Kuban KC, Leviton A. Cerebral palsy. *N Engl J Med*. 1994; 330:188–195. [PubMed: 8264743]

2. Babcock MA, Kostova FV, Ferriero DM, et al. Injury to the preterm brain and cerebral palsy: clinical aspects, molecular mechanisms, unanswered questions, and future research directions. *J Child Neurol.* 2009; 24:1064–1084. [PubMed: 19745084]
3. Mwaniki MK, Atieno M, Lawn JE, Newton CR. Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review. *Lancet.* 2012; 379:445–452. [PubMed: 22244654]
4. Koman LA, Smith BP, Shilt JS. Cerebral palsy. *Lancet.* 2004; 363:1619–1631. [PubMed: 15145637]
5. Hunt RW, Neil JJ, Coleman LT, et al. Apparent diffusion coefficient in the posterior limb of the internal capsule predicts outcome after perinatal asphyxia. *Pediatrics.* 2004; 114:999–1003. [PubMed: 15466097]
6. Thomas B, Eyssen M, Peeters R, et al. Quantitative diffusion tensor imaging in cerebral palsy due to periventricular white matter injury. *Brain.* 2005; 128:2562–2577. [PubMed: 16049045]
7. Sanger TD. Pathophysiology of pediatric movement disorders. *J Child Neurol.* 2003; 18(1 suppl):S9–S24. [PubMed: 13677568]
8. Laura LD, Deborah G-S. Assessment and treatment of movement disorders in children with cerebral palsy. *Orthop Clin North Am.* 2010; 41:507–517. [PubMed: 20868881]
9. Futagi Y, Abe J. H-reflex study in normal children and patients with cerebral palsy. *Brain Dev.* 1985; 7:414–420. [PubMed: 4061778]
10. Roessmann U, Horwitz SJ, Kennell JH. Congenital absence of the corticospinal fibers: pathologic and clinical observations. *Neurology.* 1990; 40(3 pt 1):538–541. [PubMed: 2314599]
11. Back SA, Rivkees SA. Emerging concepts in periventricular white matter injury. *Semin Perinatol.* 2004; 28:405–414. [PubMed: 15693397]
12. Ricci D, Guzzetta A, Cowan F, et al. Sequential neurological examinations in infants with neonatal encephalopathy and low Apgar scores: relationship with brain MRI. *Neuropediatrics.* 2006; 37:148–153. [PubMed: 16967366]
13. Brody BA, Kinney HC, Kloban AS, Gilles FH. Sequence of central nervous system myelination in human infancy. I An autopsy study of myelination. *J Neuropathol Exp Neurol.* 1987; 46:283–301. [PubMed: 3559630]
14. de Vries LS, van Haastert IC, Benders MJ, Groenendaal F. Myth: cerebral palsy cannot be predicted by neonatal brain imaging. *Semin Fetal Neonatal Med.* 2011; 16:279–287. [PubMed: 21636334]
15. Silbereis JC, Huang EJ, Back SA, Rowitch DH. Towards improved animal models of neonatal white matter injury associated with cerebral palsy. *Dis Model Mech.* 2010; 3:678–688. [PubMed: 21030421]
16. Meier C, Middelani J, Wasielewski B, et al. Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. *Pediatr Res.* 2006; 59:244–249. [PubMed: 16439586]
17. Thoresen M, Hobbs CE, Wood T, et al. Cooling combined with immediate or delayed xenon inhalation provides equivalent long-term neuroprotection after neonatal hypoxia-ischemia. *J Cereb Blood Flow Metab.* 2009; 29:707–714. [PubMed: 19142190]
18. Jacobson Misbe EN, Richards TL, McPherson RJ, et al. Perinatal asphyxia in a nonhuman primate model. *Dev Neurosci.* 2011; 33:210–221. [PubMed: 21659720]
19. Derrick M, Luo NL, Bregman JC, et al. Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: a model for human cerebral palsy? *J Neurosci.* 2004; 24:24–34. [PubMed: 14715934]
20. Tan S, Drobyshevsky A, Jilling T, et al. Model of cerebral palsy in the perinatal rabbit. *J Child Neurol.* 2005; 20:972–979. [PubMed: 16417845]
21. Buser JR, Segovia KN, Dean JM, et al. Timing of appearance of late oligodendrocyte progenitors coincides with enhanced susceptibility of preterm rabbit cerebral white matter to hypoxia-ischemia. *J Cereb Blood Flow Metab.* 2010; 30:1053–1065. [PubMed: 20068573]
22. Drobyshevsky A, Derrick M, Wyrwicz AM, et al. White matter injury correlates with hypertonia in an animal model of cerebral palsy. *J Cereb Blood Flow Metab.* 2007; 27:270–281. [PubMed: 16736047]

23. Drobyshevsky A, Song SK, Gamkrelidze G, et al. Developmental changes in diffusion anisotropy coincide with immature oligodendrocyte progression and maturation of compound action potential. *J Neurosci.* 2005; 25:5988–5997. [PubMed: 15976088]
24. Arteni NS, Salgueiro J, Torres I, et al. Neonatal cerebral hypoxia-ischemia causes lateralized memory impairments in the adult rat. *Brain Res.* 2003; 973:171–178. [PubMed: 12738060]
25. Craig A, Ling Luo N, Beardsley DJ, et al. Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. *Exp Neurol.* 2003; 181:231–240. [PubMed: 12781996]
26. Drobyshevsky A, Derrick M, Luo K, et al. Near-term fetal hypoxia-ischemia in rabbits: MRI can predict muscle tone abnormalities and deep brain injury. *Stroke.* 2012; 43:2757–2763. [PubMed: 22829546]
27. Yakovlev, P., Lecours, A. Regional development of the brain in early life. Oxford, UK: Blackwell; 1967. The myelogenetic cycles of regional maturation in the brain; p. 3-69.
28. van Velthoven CTJ, Kavelaars A, van Bel F, Heijnen CJ. Repeated mesenchymal stem cell treatment after neonatal hypoxia-ischemia has distinct effects on formation and maturation of new neurons and oligodendrocytes leading to restoration of damage, corticospinal motor tract activity, and sensorimotor function. *J Neurosci.* 2010; 30:9603–9611. [PubMed: 20631189]
29. Drobyshevsky A, Bregman J, Storey P, et al. Serial diffusion tensor imaging detects white matter changes that correlate with motor outcome in premature infants. *Dev Neurosci.* 2007; 29:289–301. [PubMed: 17762197]
30. Back SA, Han BH, Luo NL, et al. Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. *J Neurosci.* 2002; 22:455–463. [PubMed: 11784790]
31. Kinney HC. The encephalopathy of prematurity: one pediatric neuropathologist's perspective. *Semin Pediatr Neurol.* 2009; 16:179–190. [PubMed: 19945652]
32. Back SA, Luo NL, Borenstein NS, et al. Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci.* 2001; 21:1302–1312. [PubMed: 11160401]
33. Volpe JJ. Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *Lancet Neurol.* 2009; 8:110–124. [PubMed: 19081519]
34. Back SA, Rivkees SA. Emerging concepts in periventricular white matter injury. *Semin Perinatol.* 2004; 28:405–414. [PubMed: 15693397]
35. Back SA, Riddle A, McClure MM. Maturation-dependent vulnerability of perinatal white matter in premature birth. *Stroke.* 2007; 38:724–730. [PubMed: 17261726]
36. Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res.* 2001; 50:553–562. [PubMed: 11641446]
37. Buser JR, Maire J, Riddle A, et al. Arrested preoligodendrocyte maturation contributes to myelination failure in premature infants. *Ann Neurol.* 2012; 71:93–109. [PubMed: 22275256]
38. Inder TE, Huppi PS, Warfield S, et al. Periventricular white matter injury in the premature infant is followed by reduced cerebral cortical gray matter volume at term. *Ann Neurol.* 1999; 46:755–760. [PubMed: 10553993]
39. Inder TE, Warfield SK, Wang H, et al. Abnormal cerebral structure is present at term in premature infants. *Pediatrics.* 2005; 115:286–294. [PubMed: 15687434]
40. Pierson CR, Folkerth RD, Billiards SS, et al. Gray matter injury associated with periventricular leukomalacia in the premature infant. *Acta Neuropathol.* 2007; 114:619–631. [PubMed: 17912538]
41. Miller SP, Ferriero DM. From selective vulnerability to connectivity: insights from newborn brain imaging. *Trends Neurosci.* 2009; 32:496–505. [PubMed: 19712981]
42. Riddle A, Maire J, Gong X, et al. Differential susceptibility to axonopathy in necrotic and non-necrotic perinatal white matter injury. *Stroke.* 2012; 43:178–184. [PubMed: 22076007]
43. Volpe, JJ. Neurology of the newborn. 5th. Philadelphia, PA: Saunders/Elsevier; 2008.
44. Segovia KN, McClure M, Moravec M, et al. Arrested oligodendrocyte lineage maturation in chronic perinatal white matter injury. *Ann Neurol.* 2008; 63:520–530. [PubMed: 18393269]

45. Alix JJ, Zammit C, Riddle A, et al. Central axons preparing to myelinate are highly sensitivity to ischemic injury. *Ann Neurol*. 2012; 72:936–951. [PubMed: 23280842]
46. Alix JJP, Fern R. Glutamate receptor-mediated ischemic injury of premyelinated central axons. *Ann Neurol*. 2009; 66:682–693. [PubMed: 19938170]
47. Binderman I, Harel S, Earon Y, et al. Acute stimulation of creatine kinase activity by vitamin D metabolites in the developing cerebellum. *Biochim Biophys Acta*. 1988; 972:9–16. [PubMed: 3179337]

FIGURE 1.

Hypertonia manifests at a developmental stage before onset of myelination. (A) Postnatal day 1 (P1) kits manifest hypertonia (*insert*) before the onset of myelination. Ordinate depicts area occupied by myelinated axons measured by electron microscopy. No myelination was observed at P1, but it was observed at P11 and increased at P18. E22 = embryonic day 22; H-I = hypoxia–ischemia. (B) Major white matter (WM) fiber tracts, fimbria hippocampi (FH), anterior commissure (AC), internal capsule (IC), and corpus callosum (CC), are outlined on directionally encoded fractional anisotropy (FA) maps, obtained by in vivo magnetic resonance imaging at P1. Scale bar = 1mm. (C) Diagram depicts radial and axial components of anisotropy. Lower FA could be due either to lower axial diffusivity or to increased radial diffusivity. (D) FA was significantly decreased in the internal capsule of hypertonic kits ($n = 21$) compared to control kits ($n = 11$) at P1 ($*p < 0.04$). (E) Increased radial diffusivity component determines lower FA in hypertonic P1 kits ($*p < 0.01$), whereas axial and mean diffusivity are not different from controls. (F) A composite indicator of WM injury was the product of FA and fiber tract cross-sectional area (ordinate). This product was significantly lower in 3 of the 4 studied fiber tracts in hypertonic kits at P1 ($*p = 0.034$).

FIGURE 2.

Oligodendrocyte (OL) and axonal injury in postnatal day 1 (P1) hypertonic kits. (A, B) No significant changes were found in cell densities of any subpopulation of developing OL in internal capsule (IC, $n = 10,8$) and anterior commissure (AC, $n = 5,5$) between hypertonic and control kits at P1. PreOL = preoligodendrocyte. (C) Representative electron microphotographs of IC cross section in P1 kits. Scale bars = 500nm. (D) Quantification of axonal count in IC revealed significant decrease in unmyelinated axon density in hypertonic kits ($n = 5.5$, $*p = 0.027$). (E) Loss of smaller unmyelinated axons in hypertonia on histogram analysis of distribution of axonal size in IC. Arrow shows shift to right in hypertonic group (*solid curve*) versus controls (*dashed curve*); $*p = 0.025$. (F) Recording on perfused slice preparation of compound action potential (CAP) in internal capsule at 1

interelectrode distance. The single negative peak is attributed to unmyelinated axon response. The CAP area under the first negative peak was obtained as shown. Conduction velocity (CV), however, was calculated from regression of response latencies (*arrowheads*) at 4 interelectrode distances. (G) CV was not different between hypertonic and control groups in IC (n = 13, 8). (H) CAP areas of the single peak were also not different between hypertonic and control groups at P1 in IC (n = 13, 8).

FIGURE 3.

Structural white matter injury in hypertonic postnatal day 18 (P18) kits when myelination is present. (A) Fractional anisotropy (FA) was not significantly different between hypertonic and control P18 kits, except in corpus callosum (CC; $n = 7, 8$, $*p = 0.010$). AC = anterior commissure; FH = fimbria hippocampi; IC = internal capsule. (B) No difference was seen in directional diffusivities between groups in internal capsule. (C) The product of FA and area was lower in 3 fiber tracts ($n = 7, 8$, $*p < .05$). (D) Representative electron microscopy (EM) of IC in control and hypertonic P18 kits. Scale bars = $2\mu\text{m}$. (E, F) Axon count of IC, derived as a product of axon density from EM and corresponding tract area on magnetic resonance imaging, revealed significant loss of both unmyelinated and myelinated axons in hypertonic P18 kits after antenatal hypoxia-ischemia ($n = 6, 7$, $*p = 0.025$ in E and $*p = 0.041$ in F). (G, H) Density of unmyelinated and myelinated fibers is not significantly different between control and hypertonic groups ($n = 6, 7$).

FIGURE 4.

Hypertonic postnatal day 18 kits have similar distribution of fibers as controls in both unmyelinated (A) and myelinated (B) fibers. There is a trend of larger unmyelinated axons ($>0.3\mu\text{m}$) in the hypertonic group.

FIGURE 5.

Functional white matter changes in hypertonic postnatal day 18 kits. Compound action potential (CAP) peaks were labeled as unmyelinated (A, B), mixed (C, D), or myelinated (E, F; also see Materials and Methods). Conduction velocities (A, C, E) and peak areas (B, D, F) are presented for each fiber category: anterior commissure (AC), corpus callosum (CC), fimbria hippocampi (FH), and internal capsule (IC). Numbers in bars depict the number of recorded events satisfying the criteria for types of fibers. Hypertonic kits had less successful recordings, but if a recording was successful, the conduction velocities in unmyelinated fibers were higher in hypertonic kits in CC and lower in IC than in controls (A, $*p < .05$), and peak height was higher in hypertonic kit unmyelinated fibers in IC (B). No difference was found in conduction velocities between hypertonic and control groups in mixed and myelinated fibers (C, E), as well as in CAP areas and CAP latency intercepts.