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Spinal cord injury in hypertonic newborns after antenatal hypoxia-ischemia in a rabbit model of cerebral palsy

Running head: Spinal injury in cerebral palsy model

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ABSTRACT

While antenatal hypoxia-ischemia (H-I) is a well-established cause of brain injury, [the effects of H-I on the spinal cord remain undefined](#). This study examined whether hypertonia in rabbits [was](#) accompanied by changes in spinal architecture.

Rabbit dams underwent global fetal H-I at embryonic day 25 for 40 min. High resolution diffusion tensor imaging was performed on fixed neonatal CNS. Fractional anisotropy (FA) and regional volumetric measurements were compared between kits with and without hypertonia after H-I and sham controls using Tract Based Spatial Statistics.

Hypertonic kits [showed evidence of damage from hypoxia not only in the brain, but in spinal cord as well](#). Hypertonic kits showed reduced FA and thickness in corticospinal tracts, external capsule, fimbria, and in white and gray matter of both cervical and lumbar spinal cord. Dorsal white matter of the spinal cord was the exception, where [there was thickening and increased FA in hypertonic kits](#). Direct damage to the spinal cord was demonstrated in a subset of dams imaged during H-I with a 3T magnetic resonance scanner, where apparent diffusion coefficient in fetal spinal cords acutely decreased [during hypoxia](#). Hypertonic kits showed [subsequent decreases in](#) lumbar motoneuron counts and extensive TUNEL- and Fluoro-Jade C-positive labeling was present in the spinal cord 48 hours after H-I, demonstrating spinal neurodegeneration.

We speculate that global H-I causes significant loss of both spinal white and gray matter in hypertonic newborns [due to direct H-I injury to the spinal cord as well as due to upstream brain injury and consequent loss of descending projections](#).

Keywords: cerebral palsy; perinatal brain injury; hypoxia-ischemia; diffusion tensor imaging

Abbreviations:

ADC – apparent diffusion coefficient

CP – cerebral palsy

DTI – diffusion tensor imaging

E – embryonic day

FA – fractional anisotropy

H-I – hypoxia-ischemia

MRI – magnetic resonance imaging

P – postnatal day

ROI – region of interest

TBSS – tract based spatial statistics

WM – white matter

INTRODUCTION

Cerebral palsy (CP) is marked by postnatal cognitive and motor deficits caused by perinatal CNS injury (Babcock et al., 2009; Volpe, 2009). Exact pathophysiological mechanisms leading to motor abnormalities including muscle spasticity and hypertonia remain incompletely understood, primarily due to heterogeneity of injury localization (de Vries and Groenendaal, 2010) and paucity of animal models that faithfully reproduce postnatal motor deficits. Another reason is that motor abnormalities, including hypertonia, spasticity and dystonia, are typically revealed only later in development, when CP can reliably diagnosed (Eve, 2010). While contribution of perinatal brain injury to CP has been extensively characterized, including injury to gray matter and descending corticospinal projections, both in humans and animal models (Volpe, 2001, 2009), little attention has been paid to the spinal cord, its susceptibility to H-I injury, and the potential contribution to motor deficits that could arise from altered spinal circuitry.

Since by nature a global perinatal insult affects multiple areas in the CNS, it is difficult to pinpoint the exact location of injury associated with a motor deficit. Several patterns of global H-I brain injury have been described in human neonates, based on autopsy, magnetic resonance imaging (MRI) and ultrasound (de Vries and Groenendaal, 2010), including damage to the basal ganglia - thalamus, watershed regions, periventricular and subcortical white matter (WM) and cortex. One leading hypotheses is that hypertonia and spasticity in CP is caused by loss of inhibitory tone in the spinal cord due to injury of upper motor neurons or descending motor projections from brain to spinal cord (Sanger, 2003). Loss of descending control results in abnormal synaptic drive of spinal motoneurons, which are activated directly by descending inputs and indirectly through patterning networks (Cahill-Rowley and Rose, 2014). Injury to ascending sensory fibers (Hoon et al., 2009) and the cortical subplate (Volpe, 2009) are also thought to contribute to motor deficits. The effect of perinatal brain injury on spinal cord development, and the possibility of concomitant injury to the spinal cord has received relatively little attention, with notable exceptions in which disruption of spinal development after early selective cortical neuronal ablation or silencing in animal models have been considered (Clowry, 2007; Han et al., 2013; Martin et al., 1999). Acute spinal cord injury was identified in asphyxiated infants (Clancy et al., 1989; Rorke, 1982; Sladky and Rorke, 1986), and after perinatal H-I, spinal cord injury can be accompanied by involvement of deep nuclear structures of basal ganglia, thalamus and brain stem (Natsume et al., 1995). Spinal involvement is accompanied clinically by hypotonia and weakness, and electrophysiologically by signs of anterior horn cell disturbance. Thus, motor deficits may be caused by injuries to broader regions of the CNS than previously thought.

While our previous studies in the rabbit model of global fetal H-I utilized a region of interest (ROI) approach based on the aforementioned hypothesis of injury localization in the brain and corticospinal tracts (Drobyshevsky et al., 2007b; Drobyshevsky et al., 2014), the primary objective of the current study was to expand characterization of WM injury across the whole CNS including the spinal cord, using group analysis without a priori assumptions. Thus, this study directly tests whether newborn kits with postnatal motor deficits show injuries predominantly in descending motor fibers or other tract types, and to examine structural changes in the spinal cord of newborn kits with motor deficits.

METHODS

The study has received approval by the Institutional Animal Care and Use Committee, and all studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Animal model of global fetal H-I and sample collection

New Zealand White pregnant rabbits (Covance, NJ) at embryonic day (E) 25 (78% term) underwent sustained uterine ischemia induced by inflation of intra-aortic balloon catheter for 40 minutes, which caused *in vivo* global fetal H-I (Derrick et al., 2004). Sham control dams underwent the same surgical procedure, including intra-aortic balloon catheter insertion, but without the balloon inflation and did not receive uterine ischemia. The dams were allowed to deliver in a nest box at term (31.5 days).

At postnatal day 1 (P1), the kits underwent neurobehavioral testing to determine the extent of motor and sensory deficits, including muscle tone measurements (Derrick et al., 2004) and were stratified into three groups: hypertonic (muscle tone score >2 at least in one limb, $n=9$, 6 males and 5 females), non hypertonic after H-I (muscle tone score $=2$, $n=8$, 6 males, 2 females) and sham control kits ($n=9$, 5 males, 4 females). Tone was assessed by passive flexion and extension of the forelimbs and hind limbs and scored (0–4) according to the modified Ashworth scale: 0 –no increase in tone, 1 - slight increase in tone when the limb is moved in flexion or extension, 2- more marked increase in tone but limb easily flexed, 3- considerable increase in tone, passive movement difficult, 4- limb rigid in flexion or extension. One or two kits per litter were included in analysis.

Immediately after neurobehavioral testing the kits were transcardially perfused with 10% buffered formalin (Sigma, St. Louis MO), brain and spinal cord removed and post-fixed in the same fixative for 1-2 weeks.

MRI of ex vivo brains and spinal cords

Fixed P1 brains and spinal cords were rehydrated in phosphate buffered saline (PBS) for 24 hours before imaging and immersed in non-aqueous media (fomblin Y, Sigma Aldrich, MO). Imaging was performed on a 14.1 T Bruker Avance imaging spectrometer (Bruker, Billerica, MA). A 20-mm resonator was used to image brain samples and a 10 mm resonator was used for spinal cord samples.

Diffusion tensor imaging (DTI) experiments consisted of 30 non-collinear directions diffusion weighted spin echo images with $b=0$ and $1.5 \text{ ms}/\mu\text{m}^2$, $\delta/\Delta=3/7 \text{ ms}$. For brain samples, imaging parameters were TR/TE/NEX 4800/14.8/1, field of view= $2.12 \times 1.57 \text{ cm}$, matrix 142×105 , 25-37 sagittal slices 0.3 mm thick with no gap covering whole brain from olfactory bulbs to the end of cerebellum. In-plane resolution was $150 \times 150 \mu\text{m}$ and brain volumes were interpolates to isotropic $150 \mu\text{m}^3$. For spinal cord samples, imaging parameters were TR/TE/NEX 1500/16.3/2, field of view= $0.66 \times 0.24 \text{ cm}$, matrix 128×48 , 5 transverse slices 1.5 mm thick with no gap. In-plane resolution was $50 \times 50 \mu\text{m}$.

Diffusion tensor maps were calculated using multivariate linear fitting of signal attenuation from the acquired diffusion weighted images (Basser and Jones, 2002). FA maps were calculated (Basser et al., 1994) using in-house software written on Matlab (MathWorks, Natick, MA).

MR imaging of fetal H-I and samples of fetal brains at 48h after H-I

A subset of dams (n=4) were subjected to the same protocol of H-I described above while being imaged inside MRI scanner, as described in detail in (Drobyshevsky et al., 2007a). Briefly, for each imaging session the dams were sedated with intravenous infusion of fentanyl (75 $\mu\text{g/kg/hr}$) and droperidol (3.75 mg/kg/hr). MRI was performed in 3T Siemens clinical magnet using quadrature extremity coil. Single shot fast spin echo T2-weighted images were taken for anatomical reference, covering all fetuses inside dam, followed by series of diffusion weighted echo-planar images (DWI) with $b=0$ and $0.8 \text{ msec}/\mu\text{m}^2$, TR/TE = 7400/70 ms, slice thickness 4 mm, matrix 256x192, and field of view 16 cm. To monitor time course of H-I, DWI imaging was performed during the 10 min of baseline before H-I, 40 min of H-I and 20 min of reperfusion. After imaging, the catheters were removed, vessels repaired and dam recovered.

In three fetuses (2 from one dam and 1 from another), fetal head and body position was sagittal relative to the imaging plane. Sagittal position of fetuses allowed unambiguous identification of the spinal cord and the fetal brain regions on each image to ensure that it did not move throughout the imaging experiment. For those fetuses, time course of ADC was extracted from the regions of interest placed on the fetal brains and corresponding cervical spinal cords. 48 hours after H-I and MRI imaging, at E27, fetuses were delivered via laparotomy and were assigned into presumably non-hypertonic and hypertonic groups if the nadir of ADC in the fetal brain during H-I was above or below threshold value $0.83 \mu\text{m}^2/\text{msec}$, as described in (Drobyshevsky et al., 2007a). MRI imaging and ADC measurement in this study were used only to stratify tissue sample by degree of injury when motor scoring was not possible, as was the case for pre-term kits that are not viable if delivered at E25 or E27. In addition, E27 spinal cord samples were obtained from a sham operated dam.

Analysis of FA and WM thickness by tract-based spatial statistics in fixed P1 brain

For the analysis of DTI data, a cross-subject voxel-wise Tract Based Spatial Statistics analysis (TBSS) (Smith et al., 2006; Smith et al., 2007), was utilized as implemented in the FSL software (<http://www.fmrib.ox.ac.uk/fsl/>). All individual FA volumes were registered to a template, the mean FA-map was calculated and thinned to represent the mean FA skeleton.

The following algorithm was implemented in Matlab to assign WM tract thickness values to each voxel in the brain (Drobyshevsky, 2016). A pair of neighborhood voxels, having FA greater than 0.2 and an angle between first eigenvectors of the diffusion tensor less than 30 degrees was considered belonging to the same WM tract. For each voxel on a WM tract, a subset of connected voxels belonging to this tract was found on intersection of on the plane, perpendicular to the tract course. The tract thickness value was determined as a diameter of the largest circle that fits within the polygon covering all voxels on the plane. As a result of the procedure, a map of WM tracts' thicknesses was created for each animal.

For each subject, voxel data were projected from FA and tract thickness maps to the nearest voxels on the mean FA skeleton. The skeleton voxel values were assigned to the maximum of the projected voxels. The values of voxels on the common skeleton were analyzed with voxel-wise cross-subject statistical analysis utilizing a general linear model (Smith et al., 2006; Smith et al., 2007). In the case of 2 groups, as in this study, testing the contrast between the group predictors is equivalent to an unpaired t-test of the mean difference between the groups. As a result of the procedure, statistical parametric maps were created containing p-values for the voxel-wise two-sample unpaired t-tests. The results were corrected for multiple comparisons by controlling the family-wise error rate. Cluster-forming threshold $c=3$ and 3000 permutations were used for nonparametric permutation inference FSL routine. Cluster-based thresholding is a method for correction for multiple comparisons by using the null distribution of the maximum (across the image) cluster size (Friston et al., 1994). This approach detects

statistically significant clusters on the basis of the number of contiguous voxels whose voxel-wise statistic values lay above a pre-determined primary threshold.

Region of interest analysis in newborns brain and spinal cord

Brain and spinal cord images were manually segmented into ROIs as shown in Figure 1 using color encoded directional diffusivities maps, where color intensity is weighted by FA. ROI definition relied primarily on anatomical landmarks and directional information. Differences in FA between gray and white matter were much larger than variations in FA in each ROI between the experimental groups, ensuring that any bias in ROI definition due to FA changes was minimal. Areas and diffusion indexes of the selected ROIs were obtained.

Major brain white matter tracts were segmented as shown on Figure 1A-C. ROIs were placed on corpus callosum genu, internal capsule, periventricular white matter at the level of anterior commissure, cingulum and **fimbria** – at the beginning of anterior thalamus, optic tract and cerebral peduncle – at the level of midbrain 4 mm posterior anterior commissure.

Segmentation of the spinal cord is shown in Figure 1D. To ensure that the same level of spinal cord is examined, ROIs were placed on a single slice, obtained from the center of cervical and lumbar expansions, identified by the widest portion on sagittal localizer scan. Spinal cord white matter was segmented into the following regions: dorsal funiculus (Figure 1D, a), which carries ascending sensory projections in the gracilis and cuneatus fasciculus, lateral funiculus (Figure 1D, b), which carries a mixture of fibers from descending corticospinal, rubrospinal, and ascending dorsal spinocerebellar tracts and ventral funiculus (Figure 1D, c) that contains fibers of descending reticulospinal (ReST) and vestibulospinal (VST) tracts and ascending spinothalamic tract. In rabbits, the crossed corticospinal tract travels in the lateral columns of white matter and the uncrossed travels in the ventral white matter columns, similar to primates and carnivores, and unlike rodents (Armand, 1984; Butler and Hodos, 2005). An ROI containing the substantia gelatinosa in the dorsal horn was delineated from the rest of gray matter based on directional information and less color intensity due to sharply lower FA values in this region. The rest of the spinal gray matter was delineated into intermediate and ventral portions based on anatomical landmarks including the central canal and the lateral concave point (Figure 1D).

Figure 1. Regions of interest in brain and spinal cord cross sections, overlaid on directionally encoded color FA map of a control kit. Legend on the bottom of panel A represents color encoding of the predominant diffusivity direction.

A-C. ROIs in brain white matter: ac - anterior commissure, f – fimbria, cc – corpus callosum, pwm – periventricular white matter, ic – internal capsule, cg – cingulum, op – optic tract, cp – cerebral peduncle.

D. Spinal cord white matter was segmented into dorsal (a), lateral (b) and ventral (c) funiculi. Gray matter was segmented into substantia gelatinosa (d), intermediate (e) and ventral (f) regions.

Histological analysis of injury in spinal cord

Fetal spinal cords from E27 and P1 kits (n=5 per age/per experimental group) were dissected and fixed in 10% buffered formalin for at least 24 hours, cryoprotected with 20% sucrose and frozen. 20 micron cryosections were obtained from cervical and lumbar expansions.

For *in situ* fluorescent detection of DNA fragmentation (TUNEL), tissue sections were air dried on slides, hydrated in 0.01 M PBS, and permeabilized in 0.2% Triton X-100 in PBS for 5 min. An ApopTag-fluorescein *in situ* DNA fragmentation detection kit (catalog # S7110; Millipore) was used to visualize TUNEL-labeled nuclei. Slides were mounted with ProLong Gold Antifade Mountant with DAPI (ThermoFisher Scientific).

To assess neuronal degeneration, frozen sections were mounted on gelatin coated slides, air dried, immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min, rinsed for 2 min in 70% ethanol and water, incubated in 0.06% potassium permanganate solution for 10 min, and stained in 0.0001% solution of Fluoro-Jade C (Histo-Chem, AK) in 0.1% acetic acid for 10 min, dehydrated in alcohols, cleared in xylene and mounted with DPX.

For immunostaining of astroglia, primary antibodies were chicken polyclonal Anti-GFAP antibody (Abcam ab 4674, 1:200). The secondary antibodies were Goat Anti-Chicken IgY H&L (Alexa Fluor® 488) (Abcam ab150169). The sections were hydrated in 0.01 M PBS, blocked in 5% normal goat serum, incubated with the primary antibodies at 4 °C overnight, and then with the secondary antibody diluted at room temperature for 60 min and mounted with ProLong Gold Antifade Mountant with DAPI (ThermoFisher Scientific).

Stereological estimation of motor neurons' number in the spinal cord and gliosis.

Tissue was sampled at 400 µm intervals through the cervical (C4-C8) and lumbar (L4-L7) spinal cord expansions of each animal (n = 5 per group). Nissl staining was performed using cresyl violet. Motor neurons were identified by morphology (multipolar neurons with clear cytoplasm, soma diameter >20 µm and distinguishable nucleus) and location (within the ventral horns in Rexed's lamina VIII or IX) and counted bilaterally. An optical fractionator probe in StereoInvestigator (MBF Bioscience, Williston, VT, USA) was used to obtain an unbiased estimate of the total number of motor neurons by a stereological formula. The cells were counted from five sections under a 10x objective with a counting grid 50x50 µm², sampling grid 400x400 µm². The Gundersen coefficient of error of the stereological estimation for each animal ranged from 0.05 to 0.15.

Numbers of TUNEL- and Fluoro-Jade JC-positive cells were quantified in cervical lumbar spinal cord expansions using optical fractionator probe in StereoInvestigator. Spinal cord cross section areas were outlined on the section under 5x objective as reference space. The cells

were counted under a 20x objective with a counting grid $50 \times 50 \mu\text{m}^2$, sampling grid $400 \times 400 \mu\text{m}^2$. The Gundersen coefficient of error of the stereological estimation for each animal ranged from 0.05 to 0.12.

Gliososis was quantified as the area fraction of GFAP-labeled astrocyte somata in gray and white matter in cervical lumbar spinal cord expansions using area fraction fractionator probe in StereoInvestigator. White and gray matter regions were outlined on the section under 5x objective as reference space. Counting frame size was $200 \times 200 \mu\text{m}$, interframe interval $400 \mu\text{m}$, Cavalieri grid size $10 \times 10 \mu\text{m}$. The area fraction of glia was quantified under 20x objective as the ratio of product of the area per point and number of points hitting reference area over the product of the area per point and number of points hitting the sampled area. Sampling coefficient of error was less than 0.10.

Statistical analysis

Areas and diffusion indexes of the selected ROIs in brain and spinal cord, [cell number estimates and astroglia fractions](#) were compared between sham, non-hypertonic and hypertonic groups using one-way univariate ANOVA, followed by Tukey's post-hoc comparisons. Significance was determined at level 0.05. Relationship between cerebral peduncle FA and cross-section area, as dependent variables, and spinal gray and white matter cross-section area, as predictors, were assessed using a linear regression model. Statistical analysis was performed using Minitab 16.

RESULTS

Decrease of white and gray matter FA and tract thickness in brains of newborns with hypertonia

As a result of morphological thinning, during [the TBSS procedure](#) FA maps [of white matter bundles are reduced to and represented by](#) skeleton lines (depicted in green on Figure 2), onto which the results of statistical comparisons (p-values) are projected. Comparison between sham and hypertonic P1 kits using a FA metric revealed that voxels with significant decrease of FA in hypertonic group were present in [the splenium of the corpus callosum](#), periventricular WM, fimbria, cingulum, anterior commissure, internal capsule, optic tract. On the level of midbrain and pons, FA in the hypertonic group was significantly lower in cerebral peduncle, medial lemniscus and cerebellar WM (Figure 2A-D). There were no significant differences between sham control and non-hypertonic animals after H-I in the TBSS analysis of brain FA.

Noticeably, high anisotropy of the hippocampal and cerebral gray matter, previously reported at this young age (Drobyshevsky et al., 2005; McKinstry et al., 2002), gave rise to skeleton lines in the middle of the cortex and the cellular layer of hippocampus. FA was significantly decreased in the cerebral cortex of the hypertonic group specifically at levels corresponding to motor and sensory areas, as well as in the CA1 area of hippocampus. No significant voxels were found where FA in sham and in non-hypertonic group was less than in hypertonic group.

Comparison between sham, non-hypertonic and hypertonic P1 kits using the tract thickness metric revealed a significant decrease in WM tract thickness in [the splenium and genu of the corpus callosum](#), periventricular WM and cingulum (Figure 2E-I) in hypertonic group, similar to changes in the FA maps. There were no significant differences between sham control and non-hypertonic animals after H-I in the TBSS analysis of brain using the tract thickness metric.

Figure 2. Statistical parametric maps of comparisons between sham and hypertonic P1 kits after E25 H-I using FA (A-D) and WM tract thickness (E-I) metrics. Green skeleton lines are overlaid on gray scale FA and tract thickness map, shown on axial and horizontal sections. Statistically significant voxels with corrected p-value less 0.05, where the parameters were less in hypertonic group, are depicted in red – yellow scale for FA and in blue-light blue scale for tract thickness. Color bar indicates corrected-p-values range. Abbreviations of anatomic structures: ac – anterior commissure, cxm – cerebral cortex motor, cxs – cerebral cortex sensory, cc - corpus callosum, ic - internal capsule, sg - singulum, hp –hippocampus, **f –fimbria**, pwm – periventricular white matter, cp – cerebral peduncle, ml – medial lemniscus.

Comparing diffusivity measures using the traditional ROI approach, significant decreases in fractional anisotropy were found in fimbria, internal capsule, cerebral peduncles, periventricular WM, cingulum in hypertonic group relative to sham and non-hypertonic groups (Figure 3 A). The decrease of fractional anisotropy was due to the increase of radial diffusivity, while no difference was found in axial diffusivity between the groups. Hypertonic kits also had higher mean diffusivity in internal capsule and cerebral peduncles (Figure 3B).

Figure 3. Diffusivity measures of selected WM tracts in sham, non-hypertonic and hypertonic groups. Fractional anisotropy (A), mean (B), radial (C) and axial diffusivity (D) were measured using ROIs, placed as shown in Figure 1. Abbreviations of white matter structures are same as in Figure 2. *- $p < 0.05$

Loss of white and gray matter in spinal cords from kits with hypertonia

Cross sectional area of the dorsal, lateral and ventral WM regions were significantly smaller in the hypertonic group in both cervical and lumbar expansions (Figure 4A). A significant decrease in gray matter cross sectional area was observed in the substantia gelatinosa and ventral regions, in both cervical and lumbar expansions of hypertonic rabbits (Figure 4B). Notably, while the dorsal WM ROI area was smaller in the hypertonic group, when the area was normalized to the total cord cross-section area of the cord, the fraction of dorsal WM ROI area was significantly larger in the lumbar cord of hypertonic animals (sham vs. hypertonic cervical: 0.088 ± 0.012 vs. 0.125 ± 0.003 , $p = 0.14$ and lumbar: 0.072 ± 0.003 vs. 0.141 ± 0.010 , $p < 0.001$), indicating relative thickening of funiculi gracilis and cuneatus. Figure 4C demonstrates the clear decrease in cross sectional areas of white and gray matter, as well as relative thickening in dorsolateral WM in hypertonic kits' cords, shown with the same scale bar as for both groups.

A

C

Figure 4. Cross-section areas in spinal cords of sham and hypertonic groups in white (A) and gray (B) matter. *- $p < 0.05$

C. Fractional anisotropy maps in spinal cords of sham and hypertonic groups. All panes are in the same scale. Scale bar 1 mm. Color bar represents values for FA. Note increased FA in dorsolateral region (arrow) in hypertonic animal.

FA decreases in lateral funiculus and spinal gray matter, but increases in dorsal funiculus

Fractional anisotropy was decreased in kits with hypertonia in the lumbar lateral WM, but increased in dorsal cervical WM (Figure 5A). In gray matter, FA was decreased in kits with hypertonia in lumbar s. gelatinosa, intermediate and ventral ROIs (Figure 5B). The origin of anisotropic diffusion in spinal gray matter can be attributed to a predominantly left-right orientation of axons in the ventral horn, as shown on color encoded directional diffusivities map on Figure 5C.

Figure 5. Fractional anisotropy in white (A) and gray (B) matter in spinal cords of sham and hypertonic groups. *- $p < 0.05$

C. Directionally encoded fractional anisotropy maps in cervical (left panel) and lumbar (right panel) spinal cords of newborn P1 rabbit. Legend on the top left represents color encoding of the predominant diffusivity direction.

Apparent diffusion coefficient in spinal cord drops with hypoxia-ischemia.

To investigate the reason for gray matter loss in affected kits, H-I was performed on pregnant dams at E25 while being imaged with MRI. It was possible to reconstruct the time course of ADC during H-I in the spinal cords of 3 fetuses (Figure 6A). There was a decrease in ADC in the spinal cord during H-I, followed by almost complete recovery after 15 min of reperfusion-reoxygenation. The shape of the ADC curve in the fetal spinal cord was similar to the previously reported curve for fetal brains (Drobyshevsky et al., 2007a), however the amplitude of ADC drop in the spinal cord was less than the ADC drop in brains of the same fetuses (Figure 6B).

Figure 6. Time course of ADC changes in brain and spinal cord of E25 fetuses during H-I and reperfusion. **A.** Representative pictures of a fetus during H-I. Top left panel – T2 weighted image illustrating fetal anatomy; b-brain, s – spinal cord, h-heart. Color panel –ADC maps at different time points of H-I and reperfusion. Color bar indicates pseudo-colors, assigned to ADC values on the color maps. **B.** Averaged time course of ADC in fetal brain and cervical spinal cords, $n=3$ fetuses. Solid bar indicates duration of 40 min H-I during uterine ischemia. Dashed line indicates an ADC threshold for H-I injury resulting in hypertonia in newborns, based on a previous study (Drobyshevsky et al., 2007a).

Acute neuronal degeneration in spinal cord with hypertonia

Fetuses were stratified into those that were predicted to develop hypertonia or not to develop hypertonia by ADC MRI markers at E25 (Drobyshevsky et al., 2007a). Forty eight hours after H-I, at E27, the fetuses were delivered by c-section and spinal cord samples were obtained to assess apoptosis, neuronal degeneration and gliosis.

In the group predicted to become non-hypertonic, the majority of fetuses had no positive TUNEL (Figure 7A-B) or Fluoro-Jade C (Figure 7 D-E) cells. In a few cases, cells were observed but were restricted to dorsal horn (Figure 7A,E), in the substantia gelatinosa region. In the group predicted to develop hypertonia by ADC MRI markers, there were increased numbers of TUNEL-positive cells in the intermediate gray matter (Figure 7C), and Fluoro-Jade C-positive cells in the ventral and intermediate cord gray matter as well as in the substantia gelatinosa (Figure 7E), indicating neuronal degeneration in injured fetuses. [Stereological estimation confirmed significant increase of TUNEL-](#) (Figure 7J, $F_{2,14}=60.03$, $p<0.001$, $F_{2,14}=57.8$, $p<0.001$) and FJC -positive cell numbers (Figure 7K, $F_{2,14}=14.6$, $p=0.01$, $F_{2,14}=17.5$, $p=0.01$) in hypertonic group in cervical and lumbar expansions, correspondingly. Fluoro-Jade C-positive cell numbers in non-hypertonic kits were also higher than in controls, but less than in hypertonic

kits. Eight days after H-I at P1, there were no Fluoro-Jade C positive cells in either sham control, non-hypertonic or hypertonic cords. Notably, at E27 there were relatively few Fluoro-Jade C positive cells in the ventral horn, where motor neurons are located. Motor neurons could be readily identified as large polygonal cells on Nissl stained slices. However, stereological estimation of motor neurons' number revealed a significant decrease in total number of motor neurons in lumbar ($F_{2,14}=4.6$, $p=0.034$), but not cervical, expansion in hypertonic kits (Figure 7I).

Figure 7. Apoptosis and neuronal degeneration in cervical spinal cord 48 h after H-I on TUNEL staining (A-C), Fluoro-Jade C (D- G) and Nissl staining (H). Few TUNEL-positive cells were observed in sham or non-hypertonic animals (A, B), with more TUNEL-positive cells in intermediate zone of hypertonic fetal cords (C). High magnification (40x) insert shows TUNEL positive cells in green and nuclei DAPI stain in blue. In non-hypertonic fetal cords Fluoro-Jade positive cells were either not found (D), or restricted to dorsal horn (E). In hypertonic fetal cords Fluoro-Jade positive cells were observed in ventral and intermediate cord gray matter (arrows on F,G) and in substantia gelatinosa (arrowhead on G). In contrast, motor neurons, identified by large cell bodies on Nissl stained adjacent slices, were relatively unaffected, as (H, arrows). I. Stereological estimations of motor neuron' numbers (I), TUNEL-positive (J) and Fluoro-Jade

positive (K) cells in cervical and lumbar expansions. Motoneurons were identified by large polygonal cell bodies with Nissl staining, indicated by arrows and on higher (40x) magnification insert (H). Brackets on bar graphs indicate significant difference between the groups on post-hoc comparisons in ANOVA. Scale bars 1mm.

Astrogliosis was assessed in cervical and lumbar gray and white matter of sham, non-hypertonic and hypertonic groups at P1 (Figure 8A-F). Increased area fraction of GFAP-positive somata was found in the hypertonic group in lumbar gray matter ($F_{2,14} = 10.3$, $p=0.02$) (Figure 8G) relative to sham and to non-hypertonic kits.

Figure 8. Gliosis is shown with GFAP staining at P1 in cervical (A-C) and lumbar (D-F) spinal cords after sham or H-I. High magnification (40x) insert shows GFAP positive somata in green and nuclei DAPI stain in blue. Increased area occupied by GFAP-positive somata was observed in gray matter of hypertonic lumbar cords (G). Asterisk indicates significant increase of astroglia area fraction in lumbar cord in hypertonic kits. Scale bars 1mm

A larger extent of injury in descending corticospinal projections corresponds to a larger loss in spinal white and gray matter.

A regression analysis was used to assess the association between a loss or disruption of corticospinal connections and the loss of spinal gray and white matter in individual animals after H-I injury. There was a significant positive correlation between cross-sectional area loss in spinal white and gray matter, and the microstructural and volumetric changes in the descending corticospinal projections at the level of cerebral peduncles, using FA and cerebral peduncle cross-sectional area as predictors. The cerebral peduncles were chosen since they comprise a large bundle carrying fibers that connecting the cortex, pons and spinal cord. P-values for the regression slopes were 0.012 for FA and cervical WM area and <0.001 for all other models (Figure 9).

Figure 9. Dependence of spinal white (A,C) and gray (B,D) matter cross-sectional area loss on volumetric (A,B) and microstructural changes (C,D) and in the descending corticospinal projections. Horizontal axes represent cross-section tract area and FA, measured at the cerebral peduncles. Filled markers indicate animals with hypertonia, while hollow symbols are sham or HI no hypertonia. Regression lines for the cervical cord are solid and dashed lines are for lumbar cord.

DISCUSSION

The major finding of the study is that antenatal global hypoxic-ischemic injury results in injury to multiple WM tracts in the brain, as well as injury to the spinal cord in newborn rabbit kits with motor deficits. The study provides evidence that the spinal cord is changed in several ways: 1) acutely through injury to the gray matter caused by the H-I episode, 2) through degenerative changes in descending projections from brain to spinal cord due to upstream brain injury, and 3) through disrupted development which results in fewer motor neurons and smaller cervical and lumbar enlargements. Relative contribution and interaction of the three mechanisms to motor control deficits remains to be elucidated.

Hypoxia-ischemia causes global gray and white matter injury

Widespread reduction of FA in the CNS of newborn rabbits with hypertonia was confirmed using TBSS. Previously this had been demonstrated only in selected tracts using ROI approach (Drobyshevsky et al., 2007b). Decreased FA and reduced volume corresponded to loss of phosphorylated neurofilaments in the corpus callosum and internal capsule and was associated with hypertonia, while loss of unmyelinated fibers was found only in hypertonic kits (Drobyshevsky et al., 2014). Cortical and subcortical neuronal degeneration has also been observed in this rabbit model (Buser et al., 2010). In the current study, group voxel-wise analysis across the whole brain indicates global injury to gray and WM, including decreased FA and tract thickness particularly in pyramidal, commissural and limbic tracts (specifically corona radiata, internal capsule, cerebral peduncles; callosum and anterior commissure; fimbria, cingulum), and periventricular WM. Since the microstructural injury and loss of integrity of white matter that is assessed by diffusion weighted imaging may or may not accompany physical loss of fibers and decreased cross sectional area of a tract, we considered both tract thickness and directional diffusivities as complimentary measures of injury. This is important in the context of perinatal brain injury, since for example in hypertonic rabbits decreased FA in the internal capsule at P1 is normalized by P18 though tract area remains diminished (Drobyshevsky et al., 2014). In that study decreased cross-sectional area of WM tracts was due to axon loss but functional properties (including conduction velocity) of the preserved fibers were unaffected. Numerous studies provide evidence of decreased FA and/or increased mean diffusivity to indicate loss of integrity of corticospinal pathways (Scheck et al., 2012), while some studies indicate that volume loss of corticospinal tracts, but not diffusion metrics, correlates with the degree of functional impairment (Rha et al., 2012). In the current study both FA and tract thickness indicated widespread injury to the CNS of hypertonic rabbits.

The pattern of perinatal brain injury in the rabbit H-I model, which resulted in motor deficits in newborn rabbit kits, appears similar to the widespread WM injury detected in preterm neonates. In both rabbits and infants, involvement of motor and commissural tracts correlated with adverse neurological outcome (Duerden et al., 2015), including diffusely reduced FA in all WM tracts in preterm children with cerebral palsy (Ceschin et al., 2015). A decrease of FA in multiple WM tracts was also found in the global brain injury model of fetal cord occlusion in primates using TBSS analysis (Traudt et al., 2013). Because of the widespread injury to gray and WM that we observed, including injury to descending and ascending corticospinal tracts, abnormalities in spinal cord were anticipated. Indeed, significant loss of tissue was found not only in spinal cord WM, but also in the gray matter after antenatal H-I. Perhaps motor deficits associated with CP in children could be caused by similarly widespread injuries to the CNS.

Injury to the descending brain projections may contribute to the loss of white matter in spinal cord. In this study the loss in spinal gray and white matter was proportional to the decrease in cross-sectional area of the cerebral peduncles, a large portion of which carries corticospinal tract fibers. In human infants, decreased area of cerebral peduncles correlated with impaired dexterity (Duque et al., 2003) and injury to corticospinal tracts correlated with the level of motor impairment (Ludeman et al., 2008; Reid et al., 2016), suggesting that injuries to corticospinal tracts are the origin of motor deficits. However, it should be noted that in the rabbit model the white matter loss was found across all areas of the spinal cord, including dorsal, lateral and ventral funiculi, and cannot be attributed only to pyramidal tracts; other ascending and descending tracts were also involved. Moreover, the most significant changes in FA and cross sectional area were found in the lumbar cord expansion, while in rabbits the corticospinal projections terminate primarily in the cervical dorsal horn and do not reach the thoracic and

lumbar cord (Armand, 1984; Butler and Hodos, 2005). Thus damage to corticospinal tracts alone cannot explain the losses in spinal WM.

The role of spinal cord injury in perinatal hypoxia-ischemia

The potential contribution of spinal cord dysfunction to the genesis of abnormal neuromuscular signs following perinatal brain injury has received little attention (Volpe, 2008). Although research concerning the adverse effects of perinatal hypoxia-ischemia has focused predominantly on brain lesions, previous studies have also shown that the spinal cord may be affected. More than 25 years ago, spinal cord injury was demonstrated in human neonates after perinatal hypoxia-ischemia (Palenova et al., 1979; Rorke, 1982). In 900 consecutive autopsies performed on infants who died in the first 4 weeks of life, the neuropathologic characteristics of acute hypoxic/ischemic spinal cord injury was described in 21 infants (Sladky and Rorke, 1986). Among those infants, spinal cord infarction was a common lesion associated with prematurity. Diffuse neuronal necrosis was more typical of infants delivered at or after term, with ventromedial neurons most profoundly injured.

Spinal cord injury after H-I may contribute to the clinical picture of hypoxic-ischemic encephalopathy (Rorke, 1982) since it is accompanied by hypotonia, weakness, and decreased motoneuron excitability (Kumar et al., 2005; Prakash et al., 2005; Volpe, 2008). Spinal cord injury is often problematic to detect clinically since spinal signs are often overshadowed by cerebral signs of injury such as altered mental status and seizures. Those patients may often receive antiepileptic drugs which produce hypotonia and hyporeflexia, further masking signs of spinal injury. In a study of 18 newborns following perinatal asphyxia (Clancy et al., 1989), cellular injury in the anterior horn of the spinal cord was found in the autopsies of the expired infants. Electromyographic examinations of all 6 survivors were abnormal. As neonates, they initially presented with hypotonia or flaccidity and hyporeflexia or areflexia, and of those that survived, all developed spastic quadriplegia within 5 years. This clinical observation of muscle tone evolution corroborates with our data on near term hypoxia-ischemia in rabbits, where within the first day after injury kits are hypotonic, followed by hypertonia in hind limbs 72 hours later (Drobyshevsky et al., 2012a). Thus, newborns affected by hypoxic-ischemic encephalopathy may also have concomitant injuries to the spinal cord.

Our previous studies indicate that diffusion MRI markers are useful indicators for neural injury. Adverse motor outcomes, oxidative stress and mitochondrial dysfunction can be predicted from the acute reaction of brain cells during the during immediate reperfusion-reoxygenation period of H-I and during immediate recovery (Drobyshevsky et al., 2007a; Drobyshevsky et al., 2012b). In the current study, the decline of ADC in fetal spinal cords was similar to the decline in brain ADC. While the magnitude of the ADC drop in the spinal cord was less than in the brains, this can be explained by the insufficient spatial resolution of the MRI scan resulting in partial volume effect, suggesting that the actual magnitude of the ADC drop in spinal gray matter during H-I may be comparable to the ADC drop in the brains. When the decrease in acute ADC is taken along with the signs of neurodegeneration revealed by Fluoro-Jade and TUNEL staining, these results provide evidence of primary cellular injury in spinal cord gray matter due to global H-I insult and may explain, at least partially, decreased area of spinal gray matter in P1 newborns 7 days after H-I at E25.

Primary spinal cord gray matter injury may also explain some of the spinal WM loss. Degeneration of spinal neurons could lead to anterograde degeneration of ascending and intra-spinal connections that make up the WM. Concurrent upstream injury to gray and WM in the brain would also contribute to the loss of descending projections to the spinal cord. Signs of pre-

Wallerian degeneration have been found by ADC changes in brain stem and cervical cord in neonates with focal and diffuse perinatal H-I in cortical and subcortical structures (Groenendaal et al., 2006). Signs of spinal cord injury are not commonly observed in neonates with signs of H-I encephalopathy [undergoing MRI](#); however, this could be due to the focus of the examination. These infants typically do not receive a full spinal cord examination: the regions examined are often limited to the brain stem and beginning of the cervical cord, while the majority of the spinal cord injury is found in lumbosacral region (Sladky and Rorke, 1986).

Interestingly, abnormally high FA was found in the spinal dorsolateral WM, carrying ascending sensory fibers in fasciculus gracilis and cuneatus. Ultrastructural changes underlying the increased FA, possibly increased axonal packing, remain to be elucidated, but likely represent compensatory changes in sensory motor circuitry and dysregulation after H-I injury. Presence or absence of similar changes should be examined in the spinal cord of patients with cerebral palsy, especially in light of [the evidence that H-I injury can affect the](#) sensory system (Martin et al., 1997) and abnormal sensory and motor regulation in patients (Hoon et al., 2009; Wingert et al., 2010).

Selective distribution of neuronal injury in the spinal cord with TUNEL and Fluoro-Jade staining may be attributed to either an insufficient local blood supply (Sladky and Rorke, 1986) or selective vulnerability of certain neuronal types to H-I injury. In our study at 48 hours post-injury, predominant localization of injured cells was found in substantia gelatinosa and intermediates zone, with relative sparing in sites of motoneuron localization. In contrast, a previous report indicated predominant [injuries in the](#) anterior horn (Clancy et al., 1989), although specific cell types [were not examined, and may represent overlapping populations of neurons in humans and rabbits](#). In the current study, significantly fewer motoneurons were found in the lumbar spinal expansion. This could suggest that motoneuron loss is present but not detectable at 48 hours, or that the loss of motoneurons is due to altered development. Normally during embryonic development spinal motoneurons undergo programmed cell death in a rostral – to – caudal sequence ending around 80% of gestation in mice (Yamamoto and Henderson, 1999), a time which corresponds to the H-I injury in our rabbits at 78% gestation. If the timing of programmed cell death in the rabbit is equivalent, lumbar motoneuron pools could be undergoing this process at the time of H-I injury, and their numbers could be affected by alterations in this process. [Loss of spinal interneurons, decreased numbers of](#) motoneurons and the impairment of descending tracts may [all](#) have dramatic effects on functionality of the spinal network. Together these changes result in altered spinal inhibition (Clowry, 2007; Russ et al., 2013) and post-activation depression in patients with cerebral palsy (Achache et al., 2010) and other motor deficits.

It has been widely thought that there is a rostral-caudal progression of vulnerability to H-I injury in the neuraxis (Moore, 1986), with the cerebral cortex relatively more sensitive than the spinal cord. The current study challenges this notion: brain and spinal cord injury may occur concurrently in H-I. Myelination takes place earlier in the spinal cord than brain (Brody et al., 1987) and the spinal cord is likely to be metabolically very active during the perinatal period as corticospinal motor control develops (Martin, 2005), potentially rendering it vulnerable to H-I injury. Mechanisms of injury in the spinal cord due to global H-I injury may be similar to those of brain. Activated caspase 3 (a marker of apoptosis), CD68 (a marker of inflammation), excessive nitric oxide production and subsequent nitrotyrosine formation are all observed in spinal cord tissue after severe perinatal asphyxia in [both](#) humans (Groenendaal et al., 2008) and animal models (Dorfman et al., 2009).

This study's limitations include the relatively small number of animals in the groups and the use of fixed brain and spinal cord tissue. The high spatial resolution required for TBSS

analysis required long acquisition times. With the limited tolerance of newborn animals to long scans, especially those with perinatal brain injury, we had to resort to *ex vivo* imaging of fixed brains and spinal cords. Although FA values in normal brain tissue are considered to be essentially unchanged with fixation (Sun et al., 2003), the effect of tissue fixation on FA is not fully understood in the presence of various pathological changes (Sun et al., 2005). Another potential confound of fixation could be tissue shrinkage that may affect volumetric measurements.

Conclusions

In conclusion, the current study emphasizes the importance of spinal cord injury in perinatal H-I, which is currently overlooked as a contributing factor to motor deficits in CP. Reorganization [due to both spinal cord injury and disturbed maturation of descending and ascending connections between brain and spinal cord](#) spinal cord circuitry may result from primary H-I injury. Knowledge of the pathways leading to pathology is important for the development of adequate neuroprotective strategies, such as whole-body hypothermia (Shankaran et al., 2005) and may provide new insights to pathophysiology of motor abnormalities in cerebral palsy.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health [R01 NS091278-01A1 (AD)] and the Bill & Melinda Gates Foundation, Seattle, WA [grant ID 1119410]. Authors are grateful to Dr. Kehuan Luo for the help with animal preparation.

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