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Functional correlates of central white matter maturation in perinatal period in rabbits

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Abstract

Anisotropy indices derived from diffusion tensor imaging (DTI) are being increasingly used as biomarkers of central WM structural maturation, myelination and even functional development. Our hypothesis was that the rate of functional changes in central WM tracts directly reflects rate of changes in structural development as determined by DTI indexes.

We examined structural and functional development of four major central WM tracts with different maturational trajectories, including internal capsule (IC), corpus callosum (CC), fimbria hippocampi (FH) and anterior commissure (AC). Rabbits were chosen due to perinatal brain development being similar to humans, and four time points were studied: P1, P11, P18 and adults. Imaging parameters of structural maturation included fractional anisotropy (FA), mean and directional diffusivities derived from DTI, and T2 relaxation time. Axonal composition and degree of myelination were confirmed on electron microscopy. To assess functional maturation, conduction velocity was measured in myelinated and non-myelinated fibers by electrophysiological recordings of compound action potential in perfused brain slices.

Diffusion indices and T2 relaxation time in rabbits followed a sigmoid curve during development similar to that for humans, with active changes even at premyelination stage. The shape of the developmental curve was different between the fiber tracts, with later onset but steeper rapid phase of development in IC and FH than in CC. The structural development was not directly related to myelination or to functional development. Functional properties in projection (IC) and limbic tracts (FH) matured earlier than in associative and commissural tracts (CC and AC).

The rapid phase of changes in diffusion anisotropy and T2 relaxation time coincided with development of functional responses and myelination in IC and FH between the second and third week of postnatal development in rabbits. In these two tracts, MRI indices could serve as surrogate markers of the early stage of myelination. However, the discordance between developmental change of diffusion indices, myelination and functional properties in CC and AC cautions against equating DTI indices changes as biomarkers for myelination in all tracts.

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Keywords

brain development; white matter; diffusion tensor imaging; conduction velocity

INTRODUCTION

The advantages of MRI being a non-invasive tool for dynamic measurement of longitudinal brain development has spurred the application of advanced imaging techniques in large cohorts of infants and children, and the development of novel analytical frameworks to distinguish normal and abnormal brain development (Dean Iii et al., 2014; Dubois et al., 2014; Englander et al., 2013; Prastawa et al., 2010; Vasung et al., 2013). Diffusion tensor imaging (DTI) provides important quantitative indices of anisotropic water diffusion in white matter (WM) tracts that evolve with development (Huppi et al., 1998; Mukherjee et al., 2001; Neil et al., 1998) and reflect changes in structural composition and possibly functional properties (Dubois et al., 2008b) during fiber tract maturation. The origin of diffusion anisotropy in WM is attributed to the ordered arrangement of axonal membranes, neurofilaments, microtubules and myelin sheaths (Beaulieu, 2002), but little work has been done to understand the relative contribution of the various structural elements and tissue compartments in hindered and restricted diffusion (Assaf and Basser, 2005; Ben Bashat et al., 2005), especially during perinatal development.

Fractional anisotropy (FA) and directional diffusivities changes are being increasingly used to diagnose early motor and cognitive abnormalities in neonates and infants (Anjari et al., 2007; Dubois et al., 2014; Rose et al., 2007; van der Aa et al., 2013; van Kooij et al., 2012). WM tracts are substantially anisotropic in diffusion weighted images even in fetuses and neonates long before the onset of myelination (Wimberger, Roberts et al. 1995; Neil, Shiran et al. 1998; Drobyshevsky, Song et al. 2005). FA increases with development, with contribution from changes in both radial diffusivity and axial diffusivity (Partridge, Mukherjee et al. 2004; Gao, Lin et al. 2009; Geng, Gouttard et al. 2012). While myelination is not necessary to the origin of diffusion anisotropy in WM (Beaulieu 2002), it may contribute to the further decrease of radial diffusivity by the increase of cellular barriers for water diffusion as demonstrated in genetic (Gulani, Webb et al. 2001; Song, Sun et al. 2002) or induced (Thiessen, Zhang et al. 2013) demyelination adult animal models. It has been suggested that abnormally high radial diffusivity in the posterior limb of internal capsule may represent delayed myelination in human infants (Cowan and de Vries 2005). But, histological validation of such observations has been mostly restricted to myelin basic protein or axonal immunohistochemical markers in animal models (Drobyshevsky, Derrick et al. 2007; Junya Jito 2008; Cengiz, Uluc et al. 2011; Calabrese and Johnson 2013).

Understanding the extent of contribution of myelination to the WM anisotropy indices in infant development would help establish the timing and extent of diffusion anisotropy loss as a marker of delayed myelination. On the other hand it is important to see whether changes in directional diffusivities corresponds to appearance of fast conducting myelinated axons (Drobyshevsky et al., 2005) as this may provide a biomarker of functional maturation of WM tracts (Dubois et al., 2008b). The relationship between functional maturation, reflected

by changes in conduction velocities and compound action potential (CAP) properties and structural maturation, reflected by changes in directional diffusivities and underlying axon density and composition, may vary during developmental trajectory in different WM (Dubois et al., 2008a).

Taking advantage of the fact that different WM tracts have different developmental trajectories we investigated structural-functional relationship of WM in major central tracts. We included projection, commissural, associative and limbic tracts in our study of perinatal development from neonates to juvenile rabbit kits. What is unique with this study is the special emphasis made on accurate co-registration of MRI, functional and ultrastructural measurements. The main focus of the study was on the internal capsule (IC) since perinatal injury to cortico-spinal projections is correlated with motor deficits in humans (Rose et al., 2007) and in our animal model of global antenatal hypoxia-ischemia (Drobyshevsky et al., 2007). IC is implicated as the region where major etiopathogenetic events of human cerebral palsy (CP) take place (Laura and Deborah, 2010; Sanger, 2003).

METHODS

General plan of experiments

New Zealand White pregnant rabbits (Myrtle's Rabbits, TN) were allowed to deliver in a nest box at term (31.5 days). Rabbit kits were fed by dam and allowed to grow till P18. Newborn rabbit kits are rather immature at birth in their sensory and motor development (Hudson and Distel, 1986). Rabbit kits are born naked, with eyes not open, outer ears not developed, and poor motor coordination. By day 7 they are capable of limited oriental response to auditory stimuli and begin to open their eyes on day 9 or 10. Kits start to leave the nest when 13 to 18 days old, by which time they are able to maintain a stable body temperature and have much improved motor coordination. New Zealand rabbits sexually mature at 6 month. The study followed a longitudinal design, starting from postnatal age day 1 (P1), when kits exhibit poor motor abilities and myelination has not yet begun, through P11, when kits open eyes and show more motor abilities, to P18 when kits show adult-like motor abilities and myelination is at an advanced stage. Rabbit dams were processed to obtain adult data.

At P1, P11 and P18, the kits underwent serial *in-vivo* MRI examination. After each MRI session at P1, P11 and P18, brains from a subset of kits were extirpated for electrophysiological recordings, followed by electron microscopy. The methodology of the study was focused to reveal intricate connection between changes in microstructural organization of the maturing central WM and functional properties of the tracts. We put special emphasis to co-register structural and functional measurements, taking into account the different spatial scales of the techniques such as DTI, electron microscopy and localized CAP recording on brain slices. The order of studies and sampling procedures was arranged so that the measurement sites in WM were co-registered for MRI first, then electrophysiology and histological examinations were conducted using anatomical landmarks (Figure 1).

MRI methods

Rabbit kits were sedated with i.m. injection of a mixture of Ketamine (35 mg/kg), Xylazine (5 mg/kg), and Acepromazine (1.0 mg/kg). Animals were placed supine in a cradle heated with a water blanket at 35° C and imaged in a 9.4 T Bruker Biospec system (Bruker, Billerica, MA). The receiver coil was a standard linear Bruker rat brain size coil allowing full brain coverage in P1-P18 rabbits. Number of kits was 11 for P1, 8 for P11, 8 for P18. Three rabbit dams were imaged in the same magnet using 50 mm transceiver surface coil. A modified futility design was used to calculate the number of animals in order to detect a difference of between P1 and P11 of a difference = S.D. with $\alpha=0.05$ and power=0.85.

DTI experiments consisted of 15 non-collinear directions diffusion weighted images with TR/TE/NEX 2500/26/4 matrix 128×64, zero-padded to 128×128, $\delta = 5$ ms, $\Delta = 15$ ms, with $b=0$ and $0.8 \text{ ms}/\mu\text{m}^2$. The brain was divided in to 12 oblique coronal brain slices, positioned on a sagittal localizer scan in a way that the fifth slice was on anterior commissure and the last slice was crossing anterior edge of superior colliculus. The number of slices was kept constant for all age groups to cover the same area of cerebrum. Slice thickness was therefore variable across individual and age groups and was about 1 mm for P1, P11, 1.2 mm for P18 kits, and 1.5 mm for adults. In-plane resolution after interpolation was 0.156 mm for P1 and 0.195 mm for P11 and P18 kits. To ensure reproducible pitch angle on oblique coronal imaging sections, slices were oriented orthogonal to frontal cortex pole –pons plane as determined with the aid of multi-slice sagittal localizer scan (Figure 1). Diffusion tensor was calculated using multivariate linear fitting of signal attenuation from the acquired diffusion weighted images (Basser and Jones, 2002). Apparent diffusion coefficient (ADC), axial (first eigenvalue of the diffusion tensor) and radial (average of the second and third eigenvalues) diffusivities (Song et al., 2002), and fractional anisotropy (FA) maps were calculated (Basser et al., 1994) using in-house software written on Matlab (MathWorks, Natick, MA).

T2 relaxation time measurement was performed using spin echo multi-echo sequence with TR of 4000 ms and 8 echo times varying from 20 to 160 ms with the same geometry parameters as in DTI experiment. T2 maps were obtained by fitting mono-exponential signal decay curve in the T2-weighted images with different echo times.

High resolution sagittal T2-weighted images (RARE sequence, TR/TE/NEX 4000/90/6 matrix 256×128, zero-filled to 256×256, 1 mm slice thickness) were obtained to measure cross section areas of the commissural fiber tracts.

Region of interest analysis

Regions of interest (ROIs) of internal capsule (IC), fimbria hippocampi (FH), anterior commissure (AC) and corpus callosum (CC) were outlined using semi-automated routine written in Matlab. Single seeding voxel was manually selected by clicking on a structure of interest on a directionally color encoded FA maps (Pajevic and Pierpaoli, 1999), by an observer blinded to group assignment. ROIs were systematically placed on 5th slice for AC, 7th slice for FC, 8th slice for IC and CC in rostro-caudal direction (Fig. 1D). The ROIs boundary were outlined by a region growing procedure on a single slice using FA and fiber

orientation (Sun et al., 2003). Empirically chosen cutoff values of 0.3 for FA and 0.94 for the inner product between primary eigenvectors for neighboring voxels allowed robust segmentation of CC, IC and FI of P1-P18 rabbit kits. ROIs were transferred to the maps of DTI and T2 derived metrics and average values were obtained for each tract, as well as the cross section areas of different tracts. For CC and AC, cross section measurements ROIs were manually outlined on T2-weighted sagittal images.

Electrophysiology methods

The whole brain of control rabbits kits (7 for P1, 5 for P11, 8 for P18, and 3 adults) was quickly removed from the skull and placed into ice-cold artificial cerebrospinal fluid (ACSF), oxygenated with 95% O₂-5% CO₂, and at pH = 7.4. ACSF consisted of, in mM : NaCl 124, KCl 3, CaCl₂ 2.4, MgSO₄ 1.3, NaH₂PO₄ 1.25, NaHCO₃ 26, and glucose 10. Series of horizontal slices 400 µm thick were cut on a vibratome and contained, in order of slices superior to inferior, CC, FH, IC, and AC at the level of IC. Slices positions, recorded tracts locations and coordinate system for the further co-registration of the recorded sites with MRI data are presented on Figure 1A. The slices were allowed to recover in oxygenated ACSF at room temperature for 45-60 min before recording. The slices were transferred as needed to a recording chamber, perfused with oxygenated ACSF with 95% O₂-5% CO₂, at flow rate 1-2 mL/min and maintained at 21°C. Bipolar stimulating (constructed of twisted Teflon-coated platinum-iridium wire, diameter 0.025 mm) and recording (glass capillary extracellular microelectrode, 10 µm diameter, 1-2 MΩ, filled with 3M NaCl) electrodes were inserted in the middle portion of the CC or IC along fiber course. Recordings from IC and FH were done in the similar manner on the left and right hemispheres separately and averaged. Compound action potential in central white matter remains stable in an oxygenated perfused slice preparation for more than 10 hours, as has been shown in adult mice at 33°C (Tekkok and Goldberg, 2001). In our preliminary studies the magnitude and temporal characteristics of CAP were stable for at least 8 hours, while the actual recordings were done within 2-3 hours after 1 hour recovery period.

Compound action potential (CAP) was evoked using current steps with duration of 50 µs and an amplitude 2 mA. Stimulation amplitude was selected to be slightly below the level required for a maximum response for all age groups, since the maximum response was often difficult to reach. Recorded CAPs were amplified 1000x, digitized at 8 kHz, band pass filtered (0.3 Hz-10 kHz) and acquired using pClamp 8.1 (Axon Instruments, CA) software.

Recording was done at 4 fixed distances between electrodes, spaced between 360, 480, 600, and 720 µm for P1 and P11 kits, measured by a reticle placed in microscope eyepiece. To ensure that the responses belonged to the same fiber, recordings started from the farthest distance. Once a positive signal was obtained, subsequent recordings were obtained at progressively shorter inter-electrode distances on a straight line to the stimulating electrode. For P18 kits and adults rabbits, the inter-electrode distances were increased to 1080, 1200, 1320, and 1440 µm for better separation of waveforms appearing at these ages especially for double and triple peaks that appeared, which was elicited from faster myelinated fibers. Again, for each site the measurements started with the furthest inter-electrode distance. Once a signal was obtained and a recording obtained, three other recordings were performed

at 3 intervals in a straight line from the furthest distance to the stimulating electrode. Recordings also had to satisfy the condition of shortened latency and increasing amplitude or height, thus ensuring that all four recordings were obtained along the same fiber tract (Fig. 2). If these conditions were not satisfied, we did not analyze these recordings because of uncertainty of the recordings being from a single tract. The measurements of CV were repeated at 3 separate sites for each fiber tract by moving stimulating and recording electrodes within 1-2 mm range from the previous successful recording. The data was then averaged for each tract. This way we ensured that we got an accurate representation of the population in a major tract. Linear regression analysis between inter-electrode distance and CAP arrival time, as determined by the peak latency of the negative component of CAP, was then performed. From the linear regression line, the inverse slope and intercept determined the CAP conduction velocity (CV) and CAP latency intercept respectively. The use of linear regression thus, minimizes the error from intra-animal variability.

At the end of electrophysiological measurements, digital photographs of the slices were taken and position of the electrodes were recorded in relation to the anatomical landmarks, such as hemisphere midline and IC (Figure. 1B,C) for co-registration with of electrophysiological data with MRI, electron microscopy and OL count. To mark left and right side a small nick was made at the slice corner. Slices were fixed in 4% paraformaldehyde for 24 hours. WM tracts were then identified using characteristic striation under operation microscope, dissected out with a sharp blade and embedded in 2% agarose block in known orientation and then processed for EM.

Electron microscopy

For each fiber tract, a 0.5-mm block was cut perpendicular to the fiber course, corresponding to the position between electrophysiological electrodes in IC and processed for electron microscopy. Samples were obtained from the left IC and the number of samples was 3 at P1, 3 at P11, 6 at P18 and 3 dams. All tissue processing for EM was done with tissue embedded in agarose to preserve cross-section fiber orientation (Figure 1E). Slices were additionally post fixed in mixture of 4% paraformaldehyde and 2.5% glutaraldehyde, osmicated, dehydrated and embedded in Spurr's resin between Aclar sheets (Ted Pella Inc., Redding, CA). Sections across the fibers, 0.7 nm thick, were cut on an ultramicrotome and imaged on JEOL 1230 Transmission Electron Microscope. At least ten fields of view at 20,000 magnification were taken in random locations across the fiber tract section. Number and cross section areas of myelinated and unmyelinated axons were quantified for each tract using StereoInvestigator (v. 8.26, MBF Bioscience, Williston, VT, USA) software and fractionator and Cavalieri stereological probes. The density of myelinated and unmyelinated axons, proportion of myelinated fibers and myelinated area were determined as outcome measures. The degenerating axon were distinguished by the inclusion of electron-dense bodies or multi-lamellar membranous structures with a circular or irregular contour (Hsu et al., 2006).

Total axon count in internal capsule was estimated as a product of axonal density from EM and the tract cross section area on MRI. *In vivo* tract area by MRI was used as a more reliable and technologically feasible measure rather than area on fixed slice by EM. This

method may however slightly overestimate axonal density and absolute axonal number due to tissue shrinkage during processing for EM. The tissue shrinkage may be relatively larger in younger kits due to the larger proportion of extracellular space.

Statistical Analysis

Values are expressed as Mean $\pm$ SEM. Longitudinal data were analyzed with repeated measure ANOVA. Alpha error was $\alpha=0.05$. Statistical analysis was done using SigmaStat 3.5 and MATLAB statistical toolbox.

RESULTS

Large change in diffusion weighted MRI indices reflects rapid myelination phase around P18

Microstructural maturation of central WM in rabbits was characterized by significant increase in FA (Fig. 3A). This could be predominantly explained by a decrease in radial diffusivity (Fig. 3B). Axial diffusivity did not change significantly between P1 and P18 (Fig. 3C). A composite index of WM tract maturation, defined as a product of the tracts' FA and cross-section area on MRI was determined (FAxArea). This index reflects growth of tracts and change in microstructural organization. With age, this index increased by 2-6 fold (Fig. 3D). There was also a progressive decrease of mean diffusivity (Fig. 3E), and T2 relaxation time in all studied fiber tracts with age (Fig. 3F).

The developmental changes in microstructural organization of central WM tracts on DTI corresponded to the changes in axonal composition and expansion of axonal sizes, observed on electron microscopy. The internal capsule in control P1 rabbits consisted only of unmyelinated axons (Fig. 4A). A few myelinated fibers were observed at P11 (Fig. 4B), and became abundant at P18 (Fig. 4C), with increasing density to adulthood (Fig. 4D, E). The density of unmyelinated axons progressively decreased with development (Fig. 4F), but a substantial portion of unmyelinated fibers, 83%, was retained in adulthood (Fig. 4G). Myelinated fibers proportionally occupied an increasingly large area of the IC cross section (Fig. 4H, black squares), largely contributing to the radial tract expansion (Fig. 4H, open circles). Similar increase of tract volumes was found in all studied fiber tracts.

Notably, at P18, 9-50% of the tract cross section area was myelinated (Fig. 4H) and myelinated axons comprised only 2-7% of all axons (Fig. 4G). This indicates that myelination was not complete in IC at P18, despite fully developed motor abilities of rabbit kits at this age.

The total number of axons increased from P1 to P11, remained at this level until P18 and then decreased to one-fifth of the adult number (Fig. 4G). Growth cones (Fig. 4A,C, arrows) and degenerating neurons (Fig. 4C, arrowhead) were observed at P1, P11 and at P18 reflecting possible maturational attrition of initially overabundant axons (Hsu et al., 2006). Density of degenerating neurons declined from $0.39 \pm 0.02 \mu\text{m}^{-1}$ at P1 to $0.12 \pm 0.04 \mu\text{m}^{-1}$ at P11 and to $0.03 \pm 0.01 \mu\text{m}^{-1}$ at P18, significantly correlating with age ($r=0.46$, $p=0.048$).

In spite of the decrease of total axonal count (Fig. 4G), adult IC increased in cross-section area from 0.29 ± 0.01 at P18 to $0.37 \pm 0.04 \text{ mm}^2$ partly due to the increased number and diameter expansion of myelinated fibers (Fig. 4H). Mean axonal size in myelinated axons progressively increased from 0.98 ± 0.21 at P11 to 1.06 ± 0.11 at P18, and to 1.68 ± 0.39 microns in adult. Very large axons $>3 \text{ }\mu\text{m}$ appeared at the adult stage (Fig. 5A). Distribution of unmyelinated axon diameters also progressively shifted to the right in development (Fig. 5B). Mean unmyelinated axonal diameter significantly increased from 0.23 ± 0.02 at P1 to 0.30 ± 0.01 at P11 to 0.29 ± 0.01 at P18, and significantly increased to $0.38 \pm 0.09 \text{ }\mu\text{m}$ in adults. Large unmyelinated axons larger than $0.4 \text{ }\mu\text{m}$ started appearing at later ages.

Conduction velocity of compound action potential increases with maturation in myelinated and unmyelinated fibers

Compound action potential consisted of a single peak in all studied WM tracts at P1 and in a majority of recordings at P11 (Fig. 6A). A double peak appeared in a fraction of IC and FH recordings at P11 (in 2 of 5 studied kits). At P18, the responses consisted of both single and double peaks (Fig. 6B) and the presence of multiple peaks varied between recording sites even within the same fiber tract. Double peak CAP was found in all studied fiber tracts at P18, with fewer occurrences in CC and AC (1 and 2 out of 8 studied kits respectively). Adult responses consisted of double peaks in most of tracts and some of the responses in FH had triple peaks. The appearance of double peaks has been attributed to the separation of the responses with the shorter response latencies of faster conducting myelinated fibers from the responses with longer latencies of the slower unmyelinated fibers (Reeves et al., 2005). In addition to multiple peak responses with short and long latencies, there was a population of fibers at P18 with single peak CAP but with intermediate latency (Fig. 6B).

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 for unmyelinated fiber tract CV. Similarly, the peaks with the fastest CV were considered myelinated fiber tract CV. 99th percentile of CV values of the unmyelinated and myelinated fiber responses were used to assign predominantly unmyelinated or myelinated status to single or double peak recordings. The validity of such assignment was supported by electron microscopy (Fig. 5B). The diameters of unmyelinated fibers formed single mode distribution with means 200-300 microns for all WM fiber tracts and studied ages. It was very unlikely that appearance of the second, fast peak in CAP can be attributed to a different subpopulation of unmyelinated fibers; thus, were more likely from myelinated fibers. Intermediate CV values (Fig. 6B) were considered a response of mixed fiber tracts, containing the contribution from unmyelinated and partially myelinated fibers.

CV increased with maturation both in predominantly unmyelinated (Fig 6C) and myelinated fibers (Fig. 6D). In unmyelinated fibers, CV reached almost adults values 250-300 mm/sec at P18, but in myelinated fibers the increase of CV continued after P18, especially in IC where CV doubled between P18 and adult rabbits, reflecting continuing processes of increasing myelination and axon growth. The CAP latency intercept was 1-1.5 ms for

unmyelinated axons for all tracts and ages (Fig. 6E). CAP latency intercept decreased to approximately 0.5 ms in the myelinated axons at P18 (Fig. 6F).

No significant correlations were found, however, between CV in myelinated fibers of all studied WM tracts and any of the MRI parameters, such as FA ($p=0.091$), radial ($p=0.58$) and axial ($p=0.62$) diffusivities, ADC ($p=0.9$), and T2 ($p=0.16$) in P18 kits.

Relative rate of changes in structural and functional maturation indexes from birth to adulthood

To examine whether studied MRI indices corresponded to structural and functional maturation parameters, and thus serve as non-invasive imaging markers of myelination and/or functional maturation in central WM tracts, we plotted relative change of the studied parameters with age for internal capsule (Fig. 7). Diffusion weighted indices, FA and radial diffusivity (inverted for comparison), began to change starting in premyelinating phase from P1-P11 with the largest increase around the beginning of active myelination at P11-P18 and slowed down later to adulthood (Fig. 7A). Similar to DTI indices, rate of changes in myelination indices, such as myelinated area, proportion and density of myelinated axons, as well as CV of myelinated fibers, rapidly increased from P11 to P18 (Fig. 7B).

DISCUSSION

This is the first study to examine concurrent structural and functional development of four major central fiber tracts in rabbits using diffusion tensor and T2-mapping MRI, CAP recordings and electron microscopy. The development of central WM tract was followed during first 3 weeks of life in transition from neonates with poorly developed motor and sensory abilities to juveniles when vision, hearing and locomotion are similar to adults. The main finding was that the rapid phase of changes in diffusion anisotropy and T2 relaxation time coincided with developing of functional responses and myelination in IC and FH, between the second and third week of postnatal development in rabbits.

Rapid myelination phase and diffusion indices

In the current study we observed a progressive increase of FA, and decreased mean diffusivity and T2 relaxation time with age in all studied WM tracts (Fig. 3), similar to changes reported for neonatal rabbits (D'Arceuil et al., 2005; D'Arceuil et al., 2008; Drobyshevsky et al., 2005), rodents (Calabrese and Johnson, 2013), kittens (Baratti et al., 1999) and humans (Mukherjee et al., 2001). It has long been established that myelination is not a prerequisite to the origin of diffusion anisotropy in WM (Beaulieu, 2002), and WM tracts are substantially anisotropic on diffusion weighted images even in fetuses and neonates much before the onset of myelination (Drobyshevsky et al., 2005; Wimberger et al., 1995). A period of relatively rapid increase of FA (Fig. 3A) between the 2nd and 3rd postnatal weeks in the rabbit coincided with the appearance of myelinated axons (Fig. 4A,B), and fast peaks in CAP response (Fig. 6). In rabbits, the increase in FA during this phase can be attributed to a decrease in radial diffusivity (Fig. 3B) and not in axial diffusivity, which is similar to that in humans (Mukherjee et al., 2001). However, later increase of FA to adult values in rabbits was due to a significant increase in axial diffusivity

in IC and FH and could be attributed to axon streamlining, similar to nerve maturation in rats (Takahashi et al., 2000). In humans, axial diffusivity has a trend to decrease with development (Dubois et al., 2008a; Geng et al., 2012; Mukherjee et al., 2002) but no change after 1 year of age (Gao et al., 2009).

Between second and third postnatal week in rabbits, diffusion indices and T2 values reach almost 70-80% of adult values. This period of development in rabbits is characterized by reaching important behavioral milestones: eye opening, increase of locomotive abilities, hopping and first exploratory behavior. Maturation of DTI indices continues in rabbits during at least four postnatal weeks (D'Arceuil et al., 2005). Similar, rapid phase of FA change between P12 and P24 coincides with the onset of active myelination in rats (Calabrese and Johnson, 2013; Jito et al., 2008). In kittens, this period occurs between second and fourth weeks (Baratti et al., 1999). In humans, decrease in mean diffusivity and increase in FA are rapid during the first postnatal year and slower during the second year (Geng et al., 2012; Mukherjee et al., 2001; Sadeghi et al., 2013).

Differences in structural-functional maturation rates among central WM tracts

Development of FA and T2 relaxation in the studied central WM tract in rabbits followed a sigmoid curve, similar to that previously described for humans. Active changes occurred even at a premyelination stage (Dubois et al., 2008a). The shape of the curve and the magnitude of the rapid change phase were different among the tracts (Fig 3). The rapid phase of development occurred later in IC and FH but the slope of changes was steeper than in CC, and not directly related to myelination and functional development. In contrast to IC and AC, CC had the largest FA around birth. This fact obviously has to originate from differences in intrinsic microstructural organization of the tracts, i.e. dense packing and streamlining of axons in CC, but not because of functional maturation and myelination, since the central WM in newborn rabbits is completely unmyelinated. Appearance of multiple peaks in CAP response and fast conducting fibers were observed earlier and more frequently in FH and progressively less in IC, CC and AC. This confirms the previous finding that projection (IC) and limbic tracts (FH) mature earlier than commissural and associative tracts (CC and AC) (Brody et al., 1987; Volpe, 2001). Myelination in IC at P18 was at an advanced stage but far from complete, as evidenced by the presence of responses with intermediate CVs between typical myelinated and unmyelinated values, large number of unmyelinated axons and the presence of degenerating growth cones on EM (Fig. 4C).

The difference in timing of functional maturation between central WM tracts provides some insight on the nature of diffusion indices change in early postnatal development. It would be incorrect to attribute rapid increase of FA and decrease in radial diffusivity solely to myelination since similar changes in FA and radial diffusivities occur from P11 to P18 both in CC and IC, but in contrast to IC, very few myelinated fibers were observed by electrophysiological recordings in CC even at P18. This observation confirms human DTI studies (Sadeghi et al., 2013) suggesting that FA is more related to fiber density than to myelination in CC. In contrast to rabbit and human data, the increase of myelinated fibers contents in CC between 2 and 4 postnatal weeks was the best predictor of FA growth in rats (Jito et al., 2008). Such differences in FA and maturation development can be attributed to

the difference between species. FA values in CC are very low in newborn rats, but myelination occurs faster than in rabbits. Rabbits are perinatal brain developers (Harel et al., 1985) similar to humans and unlike rodents.

Our MRI and functional data confirm that each central WM tract follows its unique developmental trajectory. Substantial efforts have been devoted to establish human normative maturation curves of diffusion indices in specific WM tracts (Dubois et al., 2008a; Geng et al., 2012; Sadeghi et al., 2013) with the hope that deviation of an individual subject data from a normative maturation curve would indicate injury (van der Aa et al., 2013) or developmental abnormalities (Prastawa et al., 2010). Such an approach definitely holds promise during rapid myelination period in the first 1-2 years of infancy when the magnitude of diffusion changes is large (Mukherjee et al., 2001; Sadeghi et al., 2013). The link between rapid diffusion indices change and early myelination phase may explain why FA measurements at 3 month infants were found to be a more reliable predictor of neurodevelopmental outcome than in neonates after neonatal stroke (van der Aa et al., 2013). This is probably because of myelination being in rapid dynamic phase at 3 months and at this time, DTI indices are more sensitive to injury. The value of diffusion indexes as biomarkers of developmental abnormalities become less diagnostic in late infancy when the diffusion changes become smaller as the dynamic phase of myelination and functional maturation of WM continues till 5-10 years (Baumann and Pham-Dinh, 2001; Fietzek et al., 2000; Lauffer and Wenzel, 1986).

Maturation of axonal composition and functional indices

There was an initial increase of axon numbers in the corticospinal tract from P1 to P11 followed later by elimination of axons (Fig 4G), leading to ~75% decrease in axonal number by adulthood. Elimination of overabundant axons has also been observed in other mammals (Schreyer and Jones, 1988; ten Donkelaar, 2000). Despite the large proportional increase of the occupied area (Fig. 4H), the actual number of myelinated axons was only 4% of all remaining axons at P11 and 18% in adults. Only 1 out of 23 axons at P1 were myelinated by adulthood in IC in the rabbit, similar to large proportion of unmyelinated axons in adult pyramidal tract in mice (Hsu et al., 2006) and humans (Lassek, 1942; Weil and Lassek, 1929). Our findings are consistent with the thinking that overabundance of descending supraspinal projections in mammals (including humans) ensures that some of these projections find their synaptic target in spinal cord and later undergo elimination and refinement (Schreyer and Jones, 1988; ten Donkelaar, 2000). A similar developmental pruning of initially overabundant connection was described for commissural fiber like CC (LaMantia and Rakic, 1990). Pruning of initially overabundant projections may even serve to change the main course of fiber bundles, as suggested by DTI tractography of anterior and posterior limbs of internal capsule in developing rabbits (D'Arceuil et al., 2008).

Axonal structural organization plays an important role in diffusion anisotropy properties during premyelination period (Dubois et al., 2008a). The amount of extracellular space visibly decreased in IC (Fig. 4A,B), while mean axonal diameter increased. The resulting increase of membrane density may contribute to the growth of FA between P1 and P11 before the onset of myelination. Large number of unmyelinated axons in IC may also

contribute to the diffusion anisotropy properties even when myelination is advanced. It should be noted that WM tissue organization is far more complex during maturation period than typically considered in analytical diffusion models (Baxter and Frank, 2013; Stanisz et al., 1997). Besides unmyelinated and myelinated compartments, it includes large proportion of growth cones, degenerating axons, large premyelinating axons, multi-lamellar structure, large amount of extracellular space (Fig.4 A-D), with different electron density and likely different relaxation times.

Structural and functional maturation in central WM

CAP recordings indicate that functional development was nearly complete in unmyelinated WM at P18 in all studied tracts (Fig. 6C), while CV in myelinated fibers continued to increase with later development (Fig. 6D). Rate of changes in myelinated CV development was the largest in IC and the smallest in CC. Similarly, in macaques, corticospinal CV at 11 month, 55 m/s, is still significantly lower than in adults, 73 m/s (Armand et al., 1994). In humans, cortico-spinal conduction matures around 5-10 years (Fietzek et al., 2000).

It has been reported that FA and radial diffusivity correlates with functional properties in the central WM in humans using visual evoked potentials (Dubois et al., 2008b). While both FA and CV increased with age in rabbits, they were not directly related to each other since we did not find significant correlation between the diffusion indices and CV of myelinated fibers at P18.

In this study we report for the first time CAP latency intercept, calculated as an intercept of the regression line between inter-electrode distance and time of CAP onset (Drobyshevsky et al., 2005). This functional property is likely to reflect passive axon membrane properties, such as axon time constant, that may indicate changes in membrane capacitance, in axon excitability, or ion channel composition. CAP latency intercept was significantly less in myelinated fibers than in unmyelinated. In accordance with our data, a study of spinal cord fibers in cats (West and Wolstencroft, 1983) showed that chronaxies of the unmyelinated axon were larger (2.06 ms) than that of myelinated axons (0.18-04 ms). In the rabbit, CAP latency intercept did not significantly differ between different tracts and across ages, suggesting that passive axon membrane properties and excitability of central WM does not significantly change in either unmyelinated or myelinated axons in development and is similar across different central WM tracts.

Fidelity of diffusion weighted MRI indices and T2 to indicate progression of myelination and functional maturation in central WM tracts

It appeared that diffusion weighted indices, FA and radial diffusivity (inverted for comparison), had the largest increase around the beginning of active myelination at P11-P18 (Fig. 7A) and closely corresponded to the rate of changes in myelination indices, such as myelinated area, proportion and density of myelinated axons, as well as CV of myelinated fibers, in this period (Fig. 7B). However, after P18, the rate of changes in DTI indices taper off, but this is not the case for myelination indices and CV. The observed difference between rates of diffusion, functional and myelination indices could be explained by the fact that at early myelination stage DTI may pick up structural changes, related not only to myelination,

but also to compacting and streamlining of axons, reduction of growth cones and degenerating axons, which all increase density of membranes and act as barriers to diffusion (Beaulieu, 2002). In addition, at the advanced myelination stage, while amount of myelin and cellular membranes continue to increase rapidly, there is also a radial expansion of axons and appearance of multiple compartments of different sizes. In advanced myelination stage, a first order diffusion tensor model obviously becomes insufficient to be adequately representative of functional and structural WM tract maturation.

Notably the changes in myelination and myelinated CV from P18 to adult were more precisely represented by changes in T2 relaxation time or even better by FA/T2 ratio (Fig. 7A), which continue to change substantially after P18. These observations suggest that T2 changes are more directly related to myelination process than DTI indices in advanced myelination stage. A combination of DTI and multicomponent T2 relaxation (Dean Iii et al., 2014; Mädler et al., 2008) may be a better imaging biomarker of functional and structural properties of central WM tracts due to the sensitivity of each method to different phases of myelination – DTI for early and T2 for advanced myelination stage.

Conclusions

The diffusion anisotropy and T2 relaxation time coincided with development of functional responses and myelination in projection and limbic tracts (IC and FH) during the rapid phase of changes in first to second week of life in rabbits. The MRI indices therefore could serve as surrogate markers of early stage myelination, functional development and injury during this period.

The diffusion tensor MRI indexes become less accurate as an index of WM myelination and functional development during the third week of life in rabbits, or juvenile period. This may have implication in evaluating DTI indexes in human infant 1-2 years and older.

The discordance between changes of diffusion indices, myelination and functional properties in commissural tracts (CC and AC) warrants caution against equating DTI indices changes as a proxy for the degree of myelination in these tracts.

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Abbreviations

CP	cerebral palsy
DTI	diffusion tensor imaging
FA	fractional anisotropy
ADC	apparent diffusion coefficient
EM	electron microscopy

WM	white matter
CC	corpus callosum
AC	anterior commissure
IC	internal capsule
FH	fimbria hippocampi
CV	conduction velocity
CAP	compound action potential

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Highlights

- White matter tracts follow unique sigmoid developmental trajectory by MRI and electrophysiology
- Rapid changes in diffusion indexes coincides with functional development in internal capsule
- Initial postnatal changes in diffusion indexes in callosum do not reflect functional maturation

Figure 1.

Placement of MRI and vibratome sections for co-registration of MRI, electrophysiological and histopathology sites. A. DTI slices (blue) were oriented orthogonal to frontal cortex pole–pons plane (solid white line) on a sagittal MRI image. Sections for electrophysiological recordings (white) were orthogonal to the DTI slices. B,C. Placement of stimulating electrodes (bipolar wire) and recording (glass pipette) electrodes were recorded with the reference to AC tract and the brain midline landmarks. Corresponding sections are shown for AC and IC recordings (B) and CC recordings (C). D. WM ROIs of interest were delineated using a semiautomatic region-growing procedure with manual placement of seed points on corresponding coronal directional color coded FA maps at P18. Scale bar 1 mm. Insert represents color coding of the fiber orientation, i.e. red indicates left-right, green indicates superior-inferior and blue indicates rostral-caudal orientation. E. After electrophysiological recordings, IC was dissected out from the slice, and sectioned across the tract for further EM studies. Scale bar 2 μ m.

Figure 2.

CAP recordings in internal capsule on perfused brain slices and determination of conduction velocity of myelinated and unmyelinated fibers. Shorter latency peak on responses with dual peak (A) was attributed to myelinated axons and longer latency peak –to unmyelinated axons. CAPs from the same fibers were registered on four distances between recording and stimulating electrodes. Linear regression fit of the response latency vs. recording distance (B) yielded indices of conduction velocity and CAP response latency intercept.

Figure 3.

Developmental maturation of WM determined by serial MRI.

A. FA developmental trajectory shown for internal capsule in bars and the other 3 tracts with symbols and lines. Rapid FA raise between P11 and P18 may be from increased myelination.

B. Developmental decrease of radial diffusivity underlies FA changes.

C. Developmental increase of FA by tract area product indicates concomitant axonal growth and expansion of tract volumes.

D. Axial diffusivity did not significantly change with development with a trend of increase in adult IC and FH and decrease in CC

E. Developmental decrease of mean diffusivity.

F. Fast decrease of T2 relaxation time with development.

Figure 4.

Ultrastructural developmental maturation of WM as determined by electron microscopy.

A-D. Representative microphotographs of cross section of internal capsule. Internal capsule at P1 (A) showed no myelination. Growth cones (arrows) were visible. Few small myelinated axons appeared at P11 (B). At P18 (C), along with partial myelination, degenerating growth cones were present (arrowheads). Myelination and axonal growth continued to adulthood (D). Scale bar 500nm on panel A, 2 μm on panels B-D.

E. Density of axons on EM showed no myelinated axons at P1, some at P11 and increased myelination at P18.

F. Density of unmyelinated axons decreased with age.

G. Total axon count in internal capsule was estimated as a product of axonal density from EM and the tract cross section area on MRI. Total IC axon count increased from P1 to P11 and then start declining due to the decrease of unmyelinated fibers. Note the large proportion of unmyelinated fibers even in adult IC.

H. IC cross section area (open circles) growth was rapid in pre-myelinated stage between P1 and P18 and was slower after P18. In contrast, rapid increase of myelinated portion of IC (black squares) continued to adulthood.

Figure 5.

Developmental changes in axonal size distribution in internal capsule determined by electron microscopy.

A. Distribution of myelinated axons at P18 (gray line) and adults (black line). Proportion of very large axons 4-6 microns in diameter was increased in adult IC.

B. Distribution of unmyelinated axon diameters shows no change in axon sizes from P1 (dashed line) to P18 (gray line). Large diameter axons appear in adults ($>0.3\mu\text{m}$, black line).

Figure 6.

Myelination and functional maturation of WM.

A. Typical CAP responses in IC at P1 and P11. Responses of all fibers at P1 and majority of recordings at P11 consisted of a single peak with long latencies and slow conduction velocities.

B. At P18, responses consisted of both single and double peaks (depicted) and sometimes triple peaks. All peaks with conduction velocities greater than the 1st percentile of the first peak in a triple peak were considered as predominantly myelinated. Correspondingly, peaks

with CV less than 99th percentile of the third peak were considered predominantly unmyelinated. Intermediate values were considered as mixed.

C. Conduction velocity in unmyelinated fibers increased up to P18 but there was no further increase in adulthood in two of the fiber tracts. IC depicted as bars and the other 3 tracts as symbols and lines.

D. Conduction velocity in myelinated fibers continued to increase after P18 to adulthood, especially in internal capsule.

E. CAP latency intercept in unmyelinated fibers did not significantly changes with age, except notable decrease in AC.

F. CAP latency intercept in myelinated axons was an order of magnitude smaller than in unmyelinated fibers in all recorded tracts.

Figure 7.

Cumulative rate of changes in non-invasive in-vivo MRI parameters (A) are shown in relation to changes of functional properties and myelination progression (B) of internal capsule. Note that MRI parameters experienced large increase between P11 and P18 (A) coinciding with the beginning of extensive myelination and then changed much less to adult values. In contrast, rapid increase in myelination and conduction velocity of myelinated fibers continued to adulthood.