Assessing prenatal white matter connectivity in commissural agenesis

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Complete or partial agenesis of the corpus callosum are rather common developmental abnormalities, resulting in a wide spectrum of clinical neurodevelopmental deficits. Currently, a significant number of these cases are detected by prenatal sonography during second trimester screening examinations. However, major uncertainties about a detailed morphological diagnosis and the clinical significance do not allow accurate prenatal counselling. Here, we were able to demonstrate the 3D connectivity of aberrant commissural tracts in 16 cases with complete and four cases with partial callosal agenesis using the foetal magnetic resonance imaging techniques of diffusion tensor imaging and tractography in utero and in vivo between gestational weeks 20 and 37. The ‘misguided’ pre-myelinated callosal axons that represent the bundle of Probst were non-invasively visualized, and they showed a degree of structural integrity similar to that of the callosal pathways of age-matched foetuses without cerebral pathologies. In two foetuses, we were able to prove, by post-mortem histology, that diffusion tensor imaging allows the depiction of the bundle of Probst, even during early stages of pre-myelination at 20 and 22 gestational weeks. In cases with partial callosal agenesis, an aberrant sigmoid-shaped bundle was prenatally depicted, confirming the findings of heterotopic interhemispheric connectivity in adults with partial callosal agenesis. In addition to the corpus callosum, other white matter pathways were also involved, including somatosensory and motor pathways that showed significantly higher fractional anisotropy values in cases with callosal agenesis compared with control subjects. A detailed prenatal assessment of abnormal white matter connectivity in cases of midline anomalies will help to explain and understand the clinical heterogeneity in these cases, taking future foetal neurological counselling strategies to a new level.

Keywords: callosal agenesis; diffusion tensor imaging; fetus; brain development; prenatal diagnosis

Abbreviations: ADC = apparent diffusion coefficient; DTI = diffusion tensor imaging
Introduction

The formation of the human forebrain commissures requires the interaction of a variety of cellular and molecular mechanisms that enable the morphogenesis of interhemispheric crossing of axons, and thus, future functional interhemispheric communication. Because of the complexity of these developmental processes, commissurisation is frequently subjected to alterations leading to a great diversity in morphological expression, ranging from hypogenesis to partial or complete forms of commissural agenesis.

Complete or partial agenesis of the corpus callosum are common developmental brain defects, with a reported combined prevalence of 0.02–0.5% (Jeret et al., 1985; Glass et al., 2008) in the general population and 2–3% in patients with mental retardation (Jeret et al., 1985).

Since the early 1980s, it has been possible to detect callosal agenesis in utero by ultrasound (Skidmore et al., 1983; Gebarski et al., 1984; Comstock et al., 1985), and it can now be reliably diagnosed by experienced sonographers by 20 gestational weeks (Vergani et al., 1994). However, prenatal diagnosis of this condition is challenged by the fact that a detailed analysis of midline structures can sometimes be difficult even for expert neurosonographers, in particular, in cases of partial callosal agenesis (Ghi et al., 2010). Moreover, the wide range of neurodevelopmental outcomes—from mild neuropsychological deficits (Lassonde et al., 1991) to severe mental retardation (Moes et al., 2009)—further complicates prenatal counselling in individual cases. In cases of complete callosal agenesis, accompanied by associated brain or body malformations, severe psychomotor deficits and intractable epileptic seizures are frequently encountered (Pilu et al., 1993; Francesco et al., 2006). In contrast, review data indicate that isolated callosal agenesis more frequently (>80%) leads to intelligence levels in the normal (Pilu et al., 1993; Vergani et al., 1994; Gupta and Lilford, 1995; Sotiriadis and Makrydimas, 2012) or normal-to-low (Moutard et al., 2012) range.

In recent years, foetal MRI has been increasingly used as an additional diagnostic tool in the assessment of pathologies of the CNS, as it offers the possibility of multiplanar depiction of subtle foetal brain structures (Schmook et al., 2010). In utero application of modern MRI techniques, such as diffusion-weighted imaging (Prayer et al., 2001; Righini et al., 2003; Kasprian et al., 2010), also enables the evaluation of maturational changes (Wimberger et al., 1995; Drobyshewsky et al., 2005) of the developing brain after 18 gestational weeks.

The technique of diffusion tensor imaging (DTI) probes the motion of protons within different tissue types, and, by measuring the amount and directionality of diffusion, it provides information on 3D tissue properties. Recently, DTI has successfully been used in utero and in vivo (Bui et al., 2006), and it has offered insights into the 3D architecture and development of major projection, commissural (Kasprian et al., 2008) and association (Mitter et al., 2011) pathways of the foetal brain.

In 1901, the psychiatrist Moritz Probst provided a detailed anatomical analysis of an acallosal and microgyric brain and described a white matter pathway—the ‘Balkenlängsbündel’—passing in an anteroposterior orientation, located medial to the lateral ventricle, cranial to the fornix and caudolateral to the cingulum (Probst, 1901). This fibre bundle is nowadays referred to as the Probst bundle and represents aberrant callosal axons, as later confirmed within mouse models (Ozaki et al., 1987; Ren et al., 2007).

Non-invasive DTI and tractography represent ideal tools with which to study certain disorders of axon guidance (Paul et al., 2007; Engle, 2010) in the developing human brain, and thus, the most appropriate in vivo imaging techniques to visualize the Probst bundles non-invasively (Lee et al., 2004; Utsunomiya et al., 2006; Wahl et al., 2009).

This controlled study pursued several aims. We wanted to evaluate the feasibility of DTI to visualize abnormal connectivity in cases of complete and partial callosal agenesis prenatally. Subsequently, results were compared with histoanatomy. Second, we aimed to assess the 3D anatomical topography of the Probst bundle between gestational weeks 20 and 37, and finally, to identify somatosensory and motor pathway changes beyond commissural connectivity.

Materials and methods

Foetuses

After obtaining informed consent for foetal MRI, 40 pregnant females (20 cases with callosal pathologies and 20 healthy cases) were included in the study. All examinations were referred by the local tertiary prenatal ultrasound centre and were clinically indicated. The study was approved by the local institutional review board. In 16 cases, foetal MRI revealed complete callosal agenesis and four cases of partial callosal agenesis. The foetal age at the initial MRI in cases with callosal agenesis, and determined by a first trimester ultrasound scan, ranged between 20 and 37 gestational weeks (mean: 27.6 ± 6.1 gestational weeks) (Table 1). Two cases with partial callosal agenesis were initially examined at 22 gestational weeks, and the other two cases at 31 and 34 gestational weeks, respectively (mean: 27.2 gestational weeks) (Table 1).

In two cases with complete callosal agenesis (Cases 3 and 6), the in utero and in vivo MRI data at 20 gestational weeks and 22 gestational weeks were correlated with a histological analysis after termination of the pregnancies at 21 gestational weeks and 22 gestational weeks. The brains of the foetuses were removed during the standard gross autopsy. The brains were fixed in buffered formalin; subsequently, brain autopsy was performed. Frontal sections of the brain and brainstem/cerebellum were harvested and embedded in paraffin according to standard protocol.

For evaluation of histomorphology, 3–5-μm thick sections were cut from the paraffin-embedded tissue blocks and were stained with haematoxylin and eosin according to standard protocol.

Immunohistochemistry used antibodies against glial fibrillary acidic protein (Dako, polyclonal, 1:3000) subsequent to 5 min proteinase K pre-treatment of tissue sections, phosphorylated neurofilament H and M (Sternerberger Monoclonals, clone SMI31, 1:50000), tubulin polymerization promoting protein (TPPP/p25, monoclonal, 1:2000). The clinical data from the examined foetuses with partial and complete callosal agenesis are outlined in Table 1.

In addition, an age-matched (maximum age difference ± 10 gestational days; mean age: 27.8 ± 5.7 gestational weeks; age range 20–37 gestational weeks) cohort of 20 foetuses without brain abnormalities detected by ultrasound or MRI was included as control subjects. In this
group, the clinical and imaging findings comprised body malformations (14/20, 70%), such as pathologies of the urogenital tract, gastroschisis, cleft lip and oligohydramnios after premature rupture of membranes (2/20, 10%). In four cases (4/20, 20%), no abnormalities were detected by prenatal imaging, and thus were considered to be normal.

**Foetal magnetic resonance imaging**

All foetal MRI examinations were performed on a 1.5 T superconducting unit (Philips Gyroscan) using a five-element phased array cardiac coil, which was wrapped around the mother’s abdomen and repositioned, if necessary, to achieve an optimal signal quality of the foetal brain. There was no sedation used. Each examination was performed within 45 min and consisted of a repertoire of optimized sequences for foetal head and body imaging. For foetal brain imaging, a set of axial, coronal and sagittal T2-weighted sequences (echo time = 140 ms, field of view = 230 mm, slice thickness: 3 mm) were acquired.

**Diffusion tensor imaging**

In periods without visible foetal movements, an axially oriented rapid echo planar diffusion tensor sequence (maximum acquisition time 1 min 50 s; 16 gradient encoding directions, b values of 0 and 700 s/mm², reconstructed asymmetric voxel size: 0.94 × 0.94 × 3 mm, field of view = 230 mm, 408 slices), optimized for foetal brain imaging, was acquired. In cases of movement artefacts, the DTI sequence was repeated up to three times until an appropriate image quality was achieved.

After visual assessment, the DTI data set with the highest quality was chosen for further post-processing, and all motion-distorted sequences with low signal-to-noise ratios were excluded from further analysis. A Philips Achieva workstation (release 12), and the diffusion registration package with 3D affine registration was used to match the available T2-weighted data sets to the fractional anisotropy colour-coded map. The ability to depict the somatosensory and

**Table 1 Characteristics of the prenatally imaged cases**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Gestational age (weeks)</th>
<th>Pathology</th>
<th>Associated malformation</th>
<th>Probst bundle</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>20</td>
<td>CCA</td>
<td>Metabolic disorder not further specified</td>
<td>Unilateral</td>
<td>Post-natal MRI + DTI proof of PB</td>
</tr>
<tr>
<td>Case 2</td>
<td>20</td>
<td>CCA</td>
<td>Lissencephaly</td>
<td>Bilateral</td>
<td>Histological proof of PB, Fig. 3</td>
</tr>
<tr>
<td>Case 3</td>
<td>20</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 4</td>
<td>22</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 5</td>
<td>22</td>
<td>CCA</td>
<td>Vermian hypoplasia</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 6</td>
<td>22</td>
<td>CCA</td>
<td>Subependymal heterotopia, hypoplastic cerebellum</td>
<td>Bilateral</td>
<td>Histological proof of PB, Fig. 4</td>
</tr>
<tr>
<td>Case 7</td>
<td>22</td>
<td>pCCA</td>
<td>Foetal brain infection</td>
<td>Bilateral + left sigmoid bundle</td>
<td>Post-natal MRI and DTI proof of PB</td>
</tr>
<tr>
<td>Case 8</td>
<td>22</td>
<td>pCCA</td>
<td>–</td>
<td>Bilateral + left sigmoid bundle</td>
<td>–</td>
</tr>
<tr>
<td>Case 9</td>
<td>27</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>Pathology proof of CCA</td>
</tr>
<tr>
<td>Case 10</td>
<td>28</td>
<td>CCA</td>
<td>Trisomy 13, hypoplastic aorta</td>
<td>Bilateral</td>
<td>Post-mortem MRI and DTI proof of PB</td>
</tr>
<tr>
<td>Case 11</td>
<td>28</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 12</td>
<td>29</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 13</td>
<td>29</td>
<td>CCA</td>
<td>Fallot’s tetralogy, hydronephrosis</td>
<td>Bilateral</td>
<td>–</td>
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<tr>
<td>Case 14</td>
<td>31</td>
<td>pCCA</td>
<td>–</td>
<td>Bilateral, no sigmoid bundle</td>
<td>–</td>
</tr>
<tr>
<td>Case 15</td>
<td>32</td>
<td>CCA</td>
<td>Pontine and cerebellar hypoplasia, mediastinal cyst</td>
<td>Unilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 16</td>
<td>34</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 17</td>
<td>34</td>
<td>pCCA</td>
<td>Schizencephaly, micrognathia, occipital meingocele, cleft lip/palate</td>
<td>Bilateral + right sigmoid bundle</td>
<td>–</td>
</tr>
<tr>
<td>Case 18</td>
<td>36</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 19</td>
<td>36</td>
<td>CCA</td>
<td>–</td>
<td>Bilateral</td>
<td>–</td>
</tr>
<tr>
<td>Case 20</td>
<td>37</td>
<td>CCA</td>
<td>Interhemispheric cyst</td>
<td>Bilateral</td>
<td>Post-natal MRI and DTI proof</td>
</tr>
</tbody>
</table>

pCCA = partial callosal agenesis; PB = Probst bundle.
motor system 3D (including the superior and posterior thalamic radiation as well as the corticospinal tracts), which appeared normal on conventional foetal magnetic resonance sequences in all examined cases, served as an internal control to guarantee reliable DTI data quality.

For the visualization of the somatosensory and motor system, a two region of interest approach was chosen, which included the posterior limb of the internal capsule and the crus cerebri, as previously described (Kasprian et al., 2008).

To visualize the Probst bundle, two coronally oriented regions of interest were placed medial to the lateral ventricles, at the level of the interventricular foramina and further posteriorly at the level of the ventricular atrium (Fig. 1).

To visualize aberrant connectivity in cases of partial callosal agenesis, two midsagittal regions of interest were placed in the region of the callosal remnants, as previously described by Tovar-Moll et al. (2007).

For tractography, the fibre assignment by continuous tracking algorithm and tracking cut-off thresholds of fractional anisotropy = 0.15, minimum fibre length of 10 mm and a variable maximum fibre angle change ranging from 27 to 35° were chosen. Random colour-coding classified the Probst bundle in blue, the somatosensory and motor trajectories in green and the fornix (Fig. 4) and the sigmoid bundle (Fig. 5) in yellow.

Statistical analysis

The SPSS statistical package 19.0 (SPSS Inc) was used for the statistical analysis. Metric data are described using mean and standard deviation. Nominal data are described using absolute frequencies and percentages. As normal and abnormal fetuses were age matched, a paired t-test was used to detect differences between their diffusion parameters. A Pearson comparison was used to assess the correlation of trajectories and diffusion measurements with gestational age. A P-value of ≤0.05 was considered to indicate a significant result.

Results

Tractography of the Probst bundle

Following the described deterministic approach in 15/20 (75%) cases with complete or partial callosal agenesis, trajectories with a distinctive topography could be visualized in 3D, and they corresponded to the anatomical descriptions of the Probst bundle. These trajectories specifically showed a fronto-occipital orientation and were located medially to the lateral ventricle (Figs 2–4).

Figure 2  Axial and oblique 3D visualizations of the somatosensory and motor trajectories (green) and the Probst bundle (blue) in cases with complete callosal agenesis (left) and the corpus callosum (blue) in normal age-matched control subjects (right) at 22 (upper row), 27 (middle row), and 34 gestational weeks (bottom row). The Probst bundles appear as massive white matter pathways, being located medial to the lateral ventricles and showing some individual heterogeneity in their 3D morphology. Brain laterality is indicated in the bottom row; l = left; r = right.
A distinct anatomical relationship with the fornix was obtained, which was located medially and caudally to the Probst bundle (yellow colour-coding on Fig. 3).

Frontally, the trajectories fanned out into the ipsilateral frontal lobes, mostly extending to the region of the superolateral frontal cortex/superior and middle frontal gyrus—if already formed. Posteriorly, the trajectories were characterized by an abrupt ending or were joined the fibres of the tapetum and passed laterally to the occipital and posterior temporal horns of the lateral ventricles (Figs 2 and 3).

For all control subjects, all parts of the corpus callosum were successfully visualized by tractography. In 19/20 (95%) of the control subjects and in all (20/20, 100%) cases with partial or complete callosal agenesis, the somatosensory and motor tracts...
were successfully depicted bilaterally. The increase in mean length of the visualized trajectories representing the Probst bundle ($P = 0.03$, Pearson) and the bilateral somatosensory and motor trajectories (left: $P = 0.03$, right: $P = 0.01$, Pearson) paralleled brain growth and correlated significantly with gestational age. Fractional anisotropy and apparent diffusion coefficient (ADC) values of the somatosensory and motor trajectories and the Probst bundle did not show a significant correlation with gestational age.

The fractional anisotropy values for right- and left-sided somatosensory and motor trajectories were found to be higher in cases with complete callosal agenesis than in age-matched control subjects (mean fractional anisotropy left somatosensory and motor tracts: $0.308 \pm 0.054$ in control subjects versus $0.382 \pm 0.084$ in cases with callosal agenesis; $P = 0.005$; paired $t$-test; Fig. 6) and mean fractional anisotropy right somatosensory and motor tracts ($0.313 \pm 0.052$ in control subjects versus $0.386 \pm 0.091$ in cases with callosal agenesis; $P = 0.008$; paired $t$-test; Fig. 6). Furthermore, lower ADC values of the right somatosensory and motor tracts in cases with callosal agenesis (mean ADC right somatosensory and motor tracts: $1.361 \pm 0.233 \times 10^{-3}$ mm$^2$/s in control subjects versus $1.219 \pm 0.160 \times 10^{-3}$ mm$^2$/s in cases with callosal agenesis; $P = 0.027$; paired $t$-test; Fig. 6) were obtained. However, similar differences in the ADC values of the left somatosensory and motor tracts did not reach statistical significance (mean ADC of left somatosensory and motor tracts: $1.320 \pm 0.218 \times 10^{-3}$ mm$^2$/s in control subjects versus $1.230 \pm 0.041 \times 10^{-3}$ mm$^2$/s in cases of callosal agenesis; $P = 0.081$; Fig. 6). Fractional anisotropy values of the Probst bundles did not differ significantly from the genu (mean fractional anisotropy $0.360 \pm 0.051$ of Probst bundles versus $0.319 \pm 0.057$ of the genu; $P = 0.059$) or the splenium (mean fractional anisotropy $0.360 \pm 0.051$ of Probst bundles versus $0.343 \pm 0.056$ of the splenium; $P = 0.103$) for normal, intact age-matched corpora callosa (Fig. 6).

Only the ADC values of the splenium were found to be significantly higher than the Probst bundle ADC values (mean ADC $1.592 \pm 0.277 \times 10^{-3}$ mm$^2$/s of Probst bundle versus $1.354 \pm 0.231 \times 10^{-3}$ mm$^2$/s of the splenium; $P = 0.017$).

In two cases (Cases 3 and 6, Table 1), in utero tractography results were correlated to a detailed histological analysis. In both cases, histology could confirm the presence of fronto-occipitally oriented, well-circumscribed, densely packed unmyelinated axonal bundles, located lateral and cranial to the fornix and lateral (and somewhat caudal) to the cortical plate of the forming cingulate gyrus, constituting the Probst bundle (Figs 3 and 4). In both cases, immunohistochemistry for glial fibrillary acidic protein demonstrated only a sparse amount of glial cell elements within this trajectory (Fig. 4), whereas neurofilament immunostaining confirmed the presence of dense axonal bundles. The axonal bundles were devoid of myelin [no detectable structures in conventional Luxol fast blue staining or in anti-MBP (myelin basic protein) immunostains].

**Tractography of the sigmoid bundle**

In four of four cases with partial callosal agenesis, tractography could visualize a sigmoid-shaped bundle connecting the right frontal pole with the left parieto-occipital region as early as 22 gestational weeks passing through the hypoplastic corpus callosum. This finding was confirmed in one case in both prenatal and post-natal follow-up examinations (Fig. 5). In 2/4 cases with partial callosal agenesis, an associated brain malformation was detected (schizencephalic cleft, ventriculomegaly with signs of foetal brain infection of unknown origin, Table 1).

**Discussion**

This MRI study aimed to visualize and non-invasively assess the abnormal connectivity of callosal trajectories in 20 non-sedated foetuses with complete or partial callosal agenesis in vivo and in utero. Using DTI, optimized for foetal brain imaging (Bui et al., 2006; Kasprian et al., 2008), the misguided callosal axonal pathways, represented by the ‘Balkenlängsbündel’ as referred to by Probst and others (Onufrowicz, 1887; Probst, 1901), were described for the first time in a larger cohort of living foetuses in 3D as early as 20 gestational weeks.

In two cases, the exact anatomical location of this distinct fibre pathway, situated cranial and lateral to the fornix and lateral and caudal to the cingulum, could be identified at 20 and 22 gestational weeks and were confirmed by the histological analysis (Figs 2 and 3). Consistent with the onset of the myelination of

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**Figure 5** Partial callosal agenesis imaged at 22 gestational weeks. Note the truncated corpus callosum, which does not extend to the level of the quadrigeminal plate. Tractography reveals a bundle passing from the right frontal to the left occipital lobe and crossing the midline in a sigmoid fashion.
human callosal axons 2 months post-natally (Flechsig, 1920; Yakovlev and Lecours, 1967; Kinney et al., 1988), myelin staining did not reveal deposits of mature myelin within the Probst bundles in one histologically and immunohistochemically examined foetal brain (Fig. 3).

Thus, this study was able to confirm the assumption that the imaging technique of in utero DTI enables us to non-invasively study the maturational processes of human white matter, before myelin is deposited by mature oligodendrocytes (Wimberger et al., 1995; Drobyshevsky et al., 2005; Kasprian et al., 2008).

Figure 6 Fractional anisotropy (FA) values for somatosensory and motor pathway connectivity showed significant differences between control foetuses and callosal agenesis foetuses (top row). These changes were reflected by lower ADC values ($10^{-3} \text{ mm}^2/\text{s}$) in the control group (middle row); however, statistical significance was apparent in only the right-sided somatosensory and motor tracts. There were no statistical differences in the fractional anisotropy values of the genu or splenium of the corpus callosum and the Probst bundle (bottom row). CCA = complete callosal agenesis.
Interestingly, microstructural changes in the callosal agenesis brain extended beyond the commissural pathways and affected the somatosensory and motor pathways, which showed higher fractional anisotropy and lower ADC values compared with those in age-matched control subjects (Fig. 6).

Finally, in utero tractography had the potential to depict the aberrant sigmoid bundle (Fig. 5), which has been recently described in post-natal cases of partial callosal agenesis (Wahl et al., 2009).

**Normal and abnormal corpus callosum development**

The human corpus callosum is unique to placental mammals and represents the largest of the human forebrain commissures. It starts to form at between 13 and 14 gestational weeks (Rakic and Yakovlev, 1968; Ren et al., 2006), as the first callosal axons originating from the neurons of the cingulate gyrus (Koester and O’Leary, 1994; Rash and Richards, 2001) pass the midline in a transient cellular structure called the massa commisuralis. This process occurs independently in time and location from the earlier (at 9 or 10 gestational weeks) formation of the anterior commissure (in the region of the ventral lamina reuniens) in an area dorsal to the fornix and hippocampal commissure (in the region of the dorsal lamina reuniens). To pass the midline, axonal path finding is guided by many molecular factors (Okada et al., 2009; Engle, 2010; Tischfield et al., 2010; Nishikimi et al., 2011) that influence axonal progression at different decision points, such as the functionally important midline glial structures [reviewed in Richards et al. (2004) and Paul et al. (2007)] and ultimately lead to connections with contralateral target neurons (Wise and Jones, 1976).

The growth and fasciculation of later-arriving axons is facilitated by the support of early pioneer axons (Koester and O’Leary, 1994; Rash and Richards, 2001; Ren et al., 2006). The characteristic shape of the corpus callosum, displaying the rostrum, genu, trunci and splenium, is essentially complete at 20 gestational weeks (Raybaud, 2010). However, the active period of axonal growth continues between 20 and 31 gestational weeks (Luttenberg, 1964; Jovanov-Milosevic et al., 2006), followed by a developmental stage, where significant retraction of callosal fibres occurs (Luttenberg, 1964; Clarke et al., 1989). This is reflected by in vivo imaging results, demonstrating that callosal growth follows a second degree polynomial function with a linear increase in callosal length during the second trimester, followed by a slower growth in the third trimester (Malinger and Zakut, 1993; Harreld et al., 2011).

The complexity of the developmental processes involved in the formation of the corpus callosum explains the numerous different genetic syndromes associated with callosal agenesis and the variety of morphological alterations of this brain structure, which has aroused the interest of generations of neuroscientists. After initial reports by Onufrowicz (1887), who interpreted the massive fronto-occipital bundle in acallosal brains as a thickening of a pre-existing physiological white matter pathway, Sachs (1892) suspected that it represented, instead, the heterotopic corpus callosum. Finally, Moriz Probst (1901) demonstrated a common anatomical origin for this ‘Balkenlängsbündel’ (longitudinal callosal bundle) and the corpus callosum of normal brains. Striking anatomical and topographical similarities of both—particularly in the frontal and occipital lobes—and the absence of the Probst bundle in normal brains, supported Probst in claiming the heterotopic nature of this pathway.

Here, we were able to demonstrate that the topography and anatomical description of the white matter pathway, which was consistently visualized by in utero tractography in a cohort of foetuses with callosal agenesis, perfectly matches the definition of Probst (Figs 2–4). It is located laterocranial to the fornix (yellow, Fig. 3), limited by the medial margin of the lateral ventricle, clearly more massive in the frontal lobes, and fans out occipitally to form the tapetum (Fig. 1).

In accordance with Probst’s original observation (Probst, 1901), the experimental tract tracing data of Ozaki and Wahlsten (1993) in foetal rats, the more recent work by Ren et al. (2007) that combined tract tracing and DTI tractography and the post-natal MRI data by Lee et al. (2004), we observed a similar topographic organization of the Probst bundle with a more disorganized structure in the ventral/caudal regions and an organized more compact organization in its dorsal segments (Fig. 3), as well as a clear distinction from the fornix (Ren et al., 2007) (Fig. 3).

The detailed and robust in utero visualization of the Probst bundle by in vivo tractography further confirms that this technique is clinically feasible in the early diagnostic assessment of the growing group of ‘disorders of axon guidance’ (Engle, 2010; Tischfield et al., 2010). It substantially widens the diagnostic capabilities of prenatal MRI, allowing for a more detailed analysis of anatomically abnormally configured or positioned white matter pathways. However, the reliability and data quality of in utero DTI is still hampered by foetal motion (Kasprian et al., 2008; Al-Mukhtar et al., 2009; Jiang et al., 2009). In 2/16 cases with complete callosal agenesis, the Probst bundle could be identified in one hemisphere only. This was mainly related to image artefacts (insufficient DTI data quality, low signal-to-noise ratio) because neither of the ipsilateral or contralateral commissures, where no typical Probst bundle is established (Loeser and Alvord, 1968; Utsunomiya et al., 1997; Brugger et al., 2007; Raybaud, 2010). The DTI data set must be checked for artefacts that would lead to incorrect negative results, to confidently exclude their presence.

**Prenatal visualization of the sigmoid bundle**

In addition to the visualization of the Probst bundle, this study demonstrated the clinical feasibility of in utero tractography for
the visualization of the aberrant commissural connectivity in cases with partial callosal agenesis (Fig. 5). As previously described by Tovar-Moll et al. (2007), and confirmed by Wahl et al. (2009), we were able to demonstrate the presence of a sigmoid-shaped pathway connecting the right frontal lobe with the left parieto-occipital region. As heterotopic connectivity seems to be common in cases of partial callosal agenesis (Wahl et al., 2009), it was not surprising to encounter this phenomenon in each of our cases with partial callosal agenesis. This finding initially supports the idea that, dependent of the defective axonal guidance cue, a wide spectrum of connectivity patterns can be encountered in partial callosal agenesis that is already present at prenatal stages—at least as early as 22 gestational weeks (Fig. 5). Moreover, the capability of foetal DTI to depict these aberrant tracts is unique and cannot be accomplished by any other diagnostic prenatal imaging technique. These initial findings promise that foetal MRI and in utero tractography will be of particular clinical value in cases where sonography reveals equivocal results regarding the presence of a complete corpus callosum. This will result in a more confident diagnosis of complete and partial callosal agenesis. Ultimately, a more specific morphological description of the encountered commissural defect will be facilitated.

Prenatal development of the Probst bundle

In post-natal imaging studies, the Probst bundle can be easily detected by its T_1 hyper- and T_2-weighted hypo-intense magnetic resonance signal characteristics at ~4 months post-natal (Barkovich and Kjos, 1988; Raybaud, 1010). As it can be reasonably assumed that the Probst bundle is not myelinated and does not show these signal properties before birth (Flechsig, 1920; Yakovlev and Lecours, 1967; Kinney et al., 1988), its presence can only be suspected by indirect signs. Thus, to date, the convincing direct visualization of the Probst bundle was only possible by post-natal imaging.

Here, we demonstrate that currently, DTI constitutes the sole in vivo imaging method by which to directly visualize this structure prenatally. In addition, we were able to prove that, even with the paucity of immunohistochemical glial staining during the early second trimester (Fig. 3), the anisotropic environment created by axons alone is sufficient to allow the 3D visualization of this pathway. This observation in the human foetal brain confirms the findings of previous animal studies (Wimberger et al., 1995; Drobshevsky et al., 2005).

Based on the ability of DTI to measure different diffusion parameters of the Probst bundle, we were able to demonstrate that the structural integrity of the Probst bundle and the developing corpus callosum are similar (Fig. 6), without significant differences from age-matched fractional anisotropy values. This further implies that both structures show a similar grade of maturity (Prayer and Prayer, 2003; Drobshevsky et al., 2005) throughout the foetal life.

The finding of significantly higher fractional anisotropy values for the somatosensory and motor trajectories in cases with complete callosal agenesis than in age-matched control subjects was surprising. There are several potential explanations for this phenomenon. First, there is a biophysical concept. Because of the absence of the corpus callosum as a massive commissural pathway, in which trajectories mainly show a left-right orientation, no crossing fibres exist that could intermingle with the corticospinal and thalamocortical connectivity. This leads to a higher degree of anisotropy in the cranio-caudal orientation, as reflected by higher fractional anisotropy values for the ‘unmasked’ somatosensory and motor pathways in cases with complete callosal agenesis. The second explanation supports an incidental observation by Sarnat (2008), who found that in some cases with callosal agenesis, callosal fibres do not contribute to the Probst bundle, but instead join corticospinal axons in the posterior limb of the internal capsule and descend with them to the spinal cord, within the uncrossed ventral funiculus, which appears to be enlarged to double or triple its normal size.

Thus, the higher fractional anisotropy values for ‘somatosensory’ fibres in cases with callosal agenesis may be related to the increased anisotropy created by a higher number of ‘mislabeled’ callosal axons following the corticospinal tract. The functions of these trajectories, however, remain unknown.

Potential impact on prognosis

If detected prenatally by ultrasound, estimating the prognosis and risk for a severe developmental deficit in cases with callosal agenesis is generally difficult. There is a high risk of severe neurodevelopmental disability in children with syndromic complete callosal agenesis and/or associated pathologies, whereas the intellectual development of children with isolated callosal agenesis can be expected to be close to normal (Pilu et al., 1993; Vergani et al., 1994; Gupta and Lilford, 1995; Moutard et al., 2012; Sotiriadis and Makrydimas, 2012). However, there is a wide clinical spectrum of this developmental defect with heterogeneity in the motor, sensory and cognitive abilities of affected individuals (Moes et al., 2009).

As the Probst bundle can be regarded as functional, with electrophysiological properties similar to those of the intact corpus callosum (Lefkowitz et al., 1991), it supposedly functionally contributes to cognitive processes in adults with complete callosal agenesis. Thus, the complete absence of the Probst bundle may indicate a severe abnormality or syndrome with a high risk of major neurodevelopmental deficits (Brugger et al., 2007). According to the high rate of associated findings in our cohort (Table 1), most of these cases were expected to show severe neurodevelopmental abnormalities. As no significant differences between cases with isolated and associated callosal agenesis in the appearance of the Probst bundle could be detected, the morphology of this structure alone may be insufficient for use as a potential predictive biomarker. More diffuse changes within the white matter architecture and association pathways (Mitter et al., 2011), which are to be identified in future studies, may serve as additional and promising findings, which will help to further optimize prognostication of severe neurodevelopmental abnormalities.

However, prediction of future cognitive abilities in isolated forms of callosal agenesis will remain difficult, as post-natal adaptive
processes in these individuals may be powerful. Their cognitive deficits are reported to be less severe than in patients with surgical callosotomy (‘split brain’) (Jeeves, 1969). In contrast, compensatory mechanisms in cases of partial callosal agenesis are less efficient, as their interhemispheric transfer deficits are greater (Dennis, 1976; Aglioti et al., 1998; Goodyear et al., 2001; Moes et al., 2009). This finding most likely relates to the phenomenon of heterotopic commissural connectivity, which, in our cases, was represented by the sigmoid bundle (Tovar-Moll et al., 2007; Wahl et al., 2009). The higher coherence of somatosensory and motor tracts in complete callosal agenesis presumably does not negatively impact motor development, as no motor deficits—except for apraxias—have been reported in individuals with callosal agenesis (Sarnat, 2008). In the future, systematic follow-up studies will have to further elucidate the functional relevance of the diverse white matter anatomy in foetuses with partial or complete callosal agenesis.

Conclusion
Currently, the techniques of DTI and tractography are unique in their ability to visualize the abnormal connectivity of the foetal brain in cases of partial and complete callosal agenesis. The unmethylated misguided callosal trajectories that form the Probst bundle can be readily depicted prenatally, and their distinct topographic relationship to other white matter pathways has been resolved in utero and in vivo. Even at early foetal developmental stages, the Probst bundle represents a highly organized and compact bundle of axons with a degree of integrity similar to that of the developing corpus callosum in control cases. In cases of partial callosal agenesis, an aberrant heterotopic commissural tract connecting the right frontal and left occipital lobes can be detected and resolved in further detail as early as the second trimester in utero. However, abnormalities in the connectivity of the foetal brain extend beyond commissural pathways alone, with differences also deployed in diffusion properties of the somatosensory and motor pathways in cases with partial and complete callosal agenesis. Therefore, future efforts will have to focus on the assessment of other white matter pathways, including the fronto-occipital, arcuate, and superior and inferior longitudinal fascicles. To further improve current prenatal foetal neurological counselling strategies, systematic and detailed clinical follow-up studies will be required to evaluate the clinical significance of the morphological heterogeneity of the misguided white matter tracts found in this population.

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