Full-length Original Research

Oligodendrocyte lineage and myelination are compromised in the gray matter of focal cortical dysplasia type IIa

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Abstract
Objectives: Focal cortical dysplasias (FCDs) are local malformations of the human neocortex and a leading cause of medically intractable epilepsy. FCDs are characterized by local architectural disturbances of the neocortex and often by a blurred gray-white matter boundary indicating abnormal white matter myelination. We have recently shown that myelination is also compromised in the gray matter of dysplastic areas, since transcripts encoding factors for oligodendrocyte differentiation and myelination are downregulated and myelin fibers appear fractured and disorganized.

Methods: Here, we characterized the gray matter–associated myelination pathology in detail by in situ hybridization, immunohistochemistry, and electron microscopy with markers for myelin, mature oligodendrocytes, and oligodendrocyte precursor cells in tissue sections of FCD IIa and control cortices. In addition, we isolated oligodendrocyte precursor cells from resected dysplastic tissue and performed proliferation assays.

Results: We show that the proportion of myelinated gray matter is similar in the dysplastic cortex to that in controls and myelinated fibers extend up to layer III. On the ultrastructural level, however, we found that the myelin sheaths of layer V axons are thinner in dysplastic specimens than in controls. In addition, the density of

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Focal cortical dysplasias (FCDs) are a frequent cause of focal epilepsy, especially in children. These cortical malformations are characterized by high seizure frequencies and pharmacoresistance. Hence, surgical resection of the lesion remains the only therapeutic option. FCDs comprise a heterogeneous group of disorders including radial and/or horizontal alterations in lamination (FCD Ia or Ib) and the occurrence of dysmorphic neurons (FCD IIa) and/or balloon cells (FCD IIb). Moreover, an abnormal white matter (WM) myelination, blurring of the gray-white matter boundary, as well as a funnel shape formation through the white matter (transmantle sign) have been reported in particular for high grade FCD IIb. The pathomechanisms underlying the FCD pathology are not yet fully understood; however, sporadic somatic mutations in the mTOR (mammalian target of rapamycin) gene and in mTOR pathway–related genes have been identified in a fraction of FCD IIa and IIb cases leading to hyperactivation of (mTOR) signaling.

The myelin sheath, created by a complex interaction between neuronal and glial cells, allows a high conduction velocity of neuronal signals over long distances. Oligodendrocytes are the myelin sheath–producing glial cells in the brain and enwrap axons with their cytoplasmic extensions. This process is intrinsically determined but modulated by the diameter of the axon and by neuronal activity. Oligodendrocytes derive from oligodendrocyte precursor cells (OPCs) and neural activity can trigger changes in myelination by influencing the proliferation of OPCs, the differentiation of oligodendrocytes, and the formation and growth of myelin sheaths.

Myelination deficiencies have been found in a number of neurologic disorders such as multiple sclerosis (MS), stroke, spinal cord injury, and cerebral palsy. Little is known about myelination disturbances in the context of FCD. Recently, we described for the first time a myelination defect in the cortical gray matter of FCD patients. So far, myelination abnormalities have been reported only for the white matter, in particular in FCD IIb cases. However, these findings were controversial: Some showed an associated loss of oligodendroglia and a correlation with epilepsy duration, but others did not. Thus, it is unclear to which extent deficits in oligodendrocyte function contribute to the FCD pathology.

In the present study, we addressed this question and characterized the morphology and ultrastructure of myelinated axons in the gray matter of adult FCD IIa cases and found that their axons are less myelinated. In addition, we examined the distribution of OPCs and of mature oligodendrocytes in the dysplastic neocortex and we isolated and cultured OPCs from resected FCD IIa and control specimens. We present evidence that in FCD IIa, OPC differentiation is severely affected, because OPCs are reduced in numbers and in their proliferation capacities.

### 2 | METHODS

#### 2.1 | Patient selection

Thirty-two surgically resected cortical specimens (FCD IIa: 14; epileptic but nondysplastic: 18) all originating from the temporal lobe (Brodmann areas 20, 21, 22, and 38) were
included in this study. Some of the above-mentioned FCD and control cases were processed and analyzed with several techniques; however, the patient cohorts were age-matched in all experiments (see Table 1).

All cases had undergone neurosurgical interventions due to pharmacoresistant epilepsy or low-grade tumors, and removal of cortical tissue was clinically warranted to achieve seizure or tumor control. In case of associated hippocampal sclerosis, the FCD was removed as part of a larger lobectomy. Pre-surgical assessment included the documentation of a detailed history, neurological examination, neuropsychological testing, MRI scanning, and noninvasive/invasive, long-term video electroencephalography (EEG) monitoring. Informed consent was obtained from all individual participants included in the study. All procedures received prior approval by the institutional review board (Ethics Committee, Medical Center - University of Freiburg) and were in accordance with the 1964 Helsinki declaration and its later amendments.

For histopathological diagnosis, all cortical and hippocampal specimens were classified on paraffin sections by the Institute of Neuropathology, Medical Center – University of Freiburg) according to Blümcke et al (2011, 2013)4,5 and Wyler et al (1992),6 respectively (Table 1).

2.2 | Tissue preparation

The tissue was collected immediately after resection in ice-cold 0.1 mol/L phosphate buffer (PB), pH 7.4, or Hanks’ Balanced Salt Solution (HBSS, Thermo Fisher). For immunohistochemistry (IHC) and in situ hybridization histochemistry (ISHH), slices were cut perpendicular to the cortex surface (~2-5 mm), fixed (4% paraformaldehyde [PFA] [w/v] in PB, 24-48 hours, 4°C), cryoprotected (30% sucrose in PB, 24-48 hours, 4°C), and stored at −80°C. For electron microscopy (EM), tissue slices (~2 mm) were fixed (4% PFA/2.0% glutaraldehyde [GA] in PB, 24 hours, 4°C) and additionally two samples were included from the −80°C stock, cut in a cryostat (60 µm) (Leica CM3050 S), and post-fixed with 4% PFA/2.0% GA in PB for 14 hours at 4°C.24

For paraffin-embedding, tissue was immersed in 4% PFA for 4h, dehydrated in increasing ethanol concentration and embedded in paraffin (Institute of Neuropathology). Paraffin blocks were cut perpendicular to the surface (3-5 µm), and sections were mounted on SuperFrost glass slides (Thermo Scientific).

2.3 | Immunohistochemistry

IHC was performed on either paraffin sections or cryosections as described before.17,25 Paraffin sections were pretreated (Tris-buffered saline with 0.1% TritonX-100, 1% bovine serum albumin [BSA, Sigma-Aldrich] and incubated overnight in the rabbit monoclonal PDGFRα [platelet-derived growth factor receptor alpha] primary antibody solution [1:500; Cell Signaling Technology] at 4°C).

Free-floating tissue sections (50µm) were pretreated (0.1% Triton X-100, 1% normal serum [NS] in PB) and incubated overnight in the primary antibody solution (4°C, mouse monoclonal anti-SMI32 [1:1000; Covance], rabbit monoclonal anti-CNPase [2’,3’-cyclic-nucleotide 3’-phosphodiesterase; 1:100; Cell Signaling Technology]). Secondary antibodies conjugated to either Cyanine (Cy) -5, -2 (1:200) or -3 (1:400; Jackson ImmunoResearch Laboratories) were used. Sections were coverslipped with ImmuMount (Thermo Scientific) or Aqueous Mount (Zytomed Systems).

2.4 | In situ hybridization histochemistry

Myelin basic protein (MBP) messenger RNA (mRNA) was investigated using digoxigenin (DIG)–labeled anti-sense riboprobes, generated from an appropriate plasmid.17

Sections were hybridized with a DIG-labeled anti-sense MBP riboprobe (55°C, overnight). DIG-labeled hybrids were detected with an anti-DIG antibody conjugated with alkaline phosphatase (Roche)17, and signal was developed with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (1 hour in the dark). Sections were coverslipped in Kaiser’s gelatin.

2.5 | Microscopy and image analysis

Photomicrographs were taken with an AxioImager microscope (Zeiss) containing digital cameras for fluorescence (MR605) and bright field (AxioCam MRc5) (both Zeiss) and were processed with Zen software (Zeiss). Composite images were scanned using the MosaiX software tool (Zeiss).

2.6 | Quantification of the myelinated area in the cortex

Images of CNPase/SMI32-labeled slices (controls: 12, FCD IIa: 9) were imported into the Fiji ImageJ software.26 In each section, two regions of interest (ROIs) were defined: ROI 1 (entire cortical gray matter) and ROI 2 (CNPase-positive area). The proportion of the myelinated cortex was calculated (myelinated area [mm²]/whole area [mm²]) × 100%).27

2.7 | Cell counting

Images of MBP mRNA-positive or PDGFRα-immunolabeled cells were imported into ImageJ software. In each section, two ROIs covering all six cortical layers were outlined.
### TABLE 1  Clinical data of FCD cases (A) and controls (B) selected for this study

| Patient ID | Sex | HS (Wyler) | Age at epilepsy onset (y) | Age at surgery (y) | Duration of epilepsy (y) | Seizure frequency | Seizure type | Histopathology | MRI findings (FCD/HS) | Methods |
|------------|-----|------------|---------------------------|-------------------|------------------------|-------------------|--------------|----------------|---------------------|---------|
| (A) Patients with FCD IIa |
| 1 | F | III | 9 | 29.9 | 20.9 | 1-2/day | SPS, CPS, SGS | IIa | FCD−, HS+ | IHC-Fr, IHC-P, PCR |
| 2 | F | no | 12 | 16.3 | 4.3 | 2/month | CPS, SGS | IIa | FCD−, HS− | ISHH, IHC-Fr, PCR |
| 3 | M | no | 11 | 15.5 | 4.5 | Several/month | A, CPS | IIa | FCD+, HS− | ISHH, IHC-Fr, PCR |
| 4 | M | no | 3 | 30.5 | 27.5 | Several/year | CPS, SGS | IIa | FCD+, HS− | ISHH, IHC-Fr, PCR |
| 5 | F | I | 2.5 | 8.7 | 6.2 | 3-5/day | SPS, CPS, SGS | IIa | FCD+, HS+ | ISHH, IHC-Fr, IHC-P, EM, PCR |
| 6 | F | II II | 6 | 31.3 | 25.3 | 4-5/month | A, CPS | IIa | FCD−, HS+ | IHC-Fr, IHC-P, EM, PCR |
| 7 | M | no | 24 | 43.1 | 19.1 | 1/week | CPS, SGS | IIa | FCD−, HS− | IHC-Fr, IHC-P, EM, PCR |
| 8 | M | n.a. | 2 | 3.9 | 1.9 | 2-7/day | MS | IIa | FCD+, HS− | CC |
| 9 | F | n.a. | 0.2 | 3.0 | 2.8 | 4/month | ATS | IIa | FCD+, HS+ | CC |
| 10 | F | III | 8 | 18.6 | 10.6 | 10/month | A, CPS | IIa | FCD+, HS+ | CC |
| 11 | F | no | 12 | 21.4 | 9.4 | 3-5/month | CPS | IIa | FCD−, HS− | IHC-P, CC, PCR |
| 12 | F | no | 12 | 22 | 33.4 | 11.4 | Several/month | A, CPS | IIa | FCD−, HS− | IHC-Fr, IHC-P, PCR |
| 13 | F | n.a. | 1.2 | 8.6 | 7.4 | 1-7/day | A, CPS | IIa | FCD+, HS+ | IHC-P |
| 14 | M | II | 0.7 | 9.3 | 8.6 | Several/week | CPS | IIa | FCD+, HS+ | IHC-Fr, IHC-P, PCR |

| (B) Control Patients without FCD |
|------------|-----|------------|---------------------------|-------------------|------------------------|-------------------|--------------|----------------|---------------------|---------|
| 1 | M | no | 17 | 18.7 | 1.7 | 2/month | CPS, SGS | DNET, grade I | HS- | ISHH, IHC-Fr, IHC-P, PCR |
| 2 | M | IV | 6 | 15.3 | 9.3 | 20/month | SPS, CPS, SGS | FCD Ia | HS+ | ISHH, IHC-Fr, IHC-P, PCR |
| 3 | M | IV | 6 | 16.7 | 10.7 | Several/day | A | Gliosis | HS+ | ISHH, IHC-Fr, IHC-P, PCR |
| 4 | F | I | 8 | 16.2 | 8.2 | 1/month | A, CPS, SGS | Ganglioglioma, grade I | HS- | IHC-Fr, IHC-P, PCR |
| 5 | M | no | 13 | 16.3 | 3.0 | 3-4/week | CPS | Ganglioglioma, grade I | HS- | ISHH, IHC-Fr, IHC-P, PCR |

(Continues)
| Patient ID | Sex | HS (Wyler/ILAE) | Age at epilepsy onset (y) | Age at surgery (y) | Duration of epilepsy (y) | Seizure frequency | Seizure type | Histopathology | MRI findings | Methods |
|------------|-----|----------------|--------------------------|-------------------|-------------------------|------------------|--------------|---------------|-------------|---------|
| 6          | F   | no             | 9                       | 12.4              | 3.4                     | 1/week           | CPS          | Cavernoma     | HS-         | ISHH, IHC-Fr, IHC-P, PCR |
| 7          | M   | IL1            | 10                      | 20.3              | 10.3                    | Several/month    | A, CPS, SGS  | Gray-white matter differentiation disturbances | HS+         | IHC-Fr, IHC-P, EM, PCR |
| 8          | M   | no             | 16                      | 16.9              | 0.9                     | 1/week           | CPS          | DNET, grade I | HS-         | IHC-Fr, EM, PCR |
| 9          | M   | n.a.           | 20                      | 55.2              | 35.2                    | 2/month          | CPS          | Gray-white matter differentiation disturbances, hippocampal gliosis | HS-         | IHC-Fr, IHC-P, EM, PCR |
| 10         | F   | no             | 33                      | 40.0              | 7.0                     | 7/month          | CPS, SGS     | Ganglioglioma, grade I | HS-         | IHC-P, EM, PCR |
| 11         | F   | no             | 4                       | 13.5              | 9.5                     | 3-4/day          | A, CPS, SGS  | Gray-white matter differentiation disturbances | HS+         | IHC-Fr, IHC-P, CC, PCR |
| 12         | F   | IL1            | n.a.                    | 28.1              | n.a.                    | n.a.             | A, CPS       | HS.           | HS+         | CC      |
| 13         | F   | no             | 3                       | 6.1               | 3.1                     | Several/year     | A            | Ganglioglioma, grade I | HS-         | CC, PCR   |
| 14         | M   | no             | 48.2                    | 55.2              | 7.0                     | Several/month    | CPS, SGS     | Meningoencephalocele | HS-         | CC      |
| 15         | M   | IL1            | 7.0                     | 35.3              | 28.3                    | 1-2/month        | A, CPS, SGS  | Gray-white matter differentiation disturbances | HS+         | CC      |
| 16         | M   | no             | 41.7                    | 48.7              | 7.0                     | 1-2/year         | CPS, SGS     | DNET, grade I | HS-         | CC      |
| 17         | M   | n.a.           | 1                       | 6.1               | 5.1                     | 3-4/day          | A, SPS, SGS  | Ganglioglioma, grade I | HS+         | IHC-Fr, PCR |
| 18         | M   | n.a.           | 12                      | 18.0              | 6.0                     | Several/month    | A, SPS, CPS, SGS | Gangliocytoma, grade I | HS-         | IHC-Fr, IHC-P, PCR |

Abbreviations: A, aura; ATS, atonic seizures; CC, cell culture; CPS, complex partial seizure; DNET, dysembryoplastic neuroepithelial tumor; EM, electron microscopy; FCD, focal cortical dysplasia; HS, hippocampal sclerosis; IHC-Fr, free-floating immunohistochemistry on frozen section; IHC-P, immunohistochemistry on paraffin section; ISHH, in situ hybridization histochemistry; MS, myoclonic seizures; n.a., not available; SGS, secondary generalized seizure; SPS, simple partial seizure; WHO, World Health Organization.
Within each, ROI-positive cells were counted using the integrated “cell counter” plugin (Cell Counter 2.2.2.jar, National Institutes of Health). Cell numbers were related to the area of the ROI to determine the cell density (cells/mm²).

2.8 | Electron microscopy

Slices (50 μm) were cut from each fixed tissue block and were stained with cresyl violet to visualize the cortical layers. The remaining tissue was cut into 1 mm slices, osmicated (2 hours, 1% osmium tetroxide in 6.86% sucrose in PB), contrasted (1% uranyl acetate in 70% ethanol, overnight, 4°C), dehydrated in graded ethanol, and embedded in epoxy resin (Durcupan ACM, Sigma-Aldrich). Ultrathin sections were cut (LEICA UC6) and collected on copper grids. Sections were analyzed with a TEM LEO 906E (Zeiss) imaged with the CCD-Camera “sharp eye” (Tröndle), and visualized with ISP software (Tröndle).

2.9 | Calculation of the g-ratio

The g-ratio is the relation of the axon diameter to the total fiber diameter.28 Photomicrographs focusing on layer V (15-25 pictures/case) were imported into ImageJ. Four hundred axons were measured (roughly 50 axons per case). Primarily axons with a distinctive circular shape were selected for this analysis.

2.10 | RNA isolation, reverse transcription, and real-time RT-qPCR analysis

The isolation of total RNA, reverse transcription, and real-time reverse transcription polymerase chain reaction (RT-qPCR) was performed as described earlier.17 The expression of mRNAs was quantified by real-time RT-qPCR on a CFX Connect Real-Time PCR System (Bio-Rad Laboratories) in the presence of SsoAdvanced Universal SYBR Green Mix (Bio-Rad). The following human-specific primer pairs were used at 70 nmol/L: S1217 and PDGFRα (forward 5′-CATGATGCTGGATTCTACTT3′, reverse 5′-TCT CTTGACTTTGACTGATG-3′). The PCR conditions were chosen as published before.17 The resulting Ct (cycle threshold) values (obtained by the CFX Manager Software, Bio-Rad Laboratories) were used to calculate relative expression levels for genes of interest.17

2.11 | Isolation of human OPCs and proliferation assay

We used a recently published protocol29 to isolate OPCs specifically from human cortical gray matter. Tissue was prepared, mechanically and enzymatically dissociated, and resuspended in OPC medium.29 The suspension was passed through a 100 μm nylon mesh strainer (BD Bioscience) and seeded on poly-L-ornithine (Sigma-Aldrich)–coated cell culture flasks. After 24 hours, the medium was supplemented with recombinant human platelet-derived growth factor AA isoform (PDGF-AA, Millipore) and cultured for 30-40 days. Cells were shaken overnight and plated on untreated plastic Petri dishes to separate OPCs.29 The total OPC number was counted and related to the fresh weight of the respective cortex tissue.

OPCs (10⁴ cells/coverslip) were seeded on poly-L-ornithine-coated coverslips (three coverslips per case) in Bottenstein and Sato30–defined medium (3 days). Proliferation capacity was determined by bromodeoxyuridine (BrdU, 50 μmol/L, Sigma Aldrich) incorporation. Cells were fixed (4% PFA, 15 min), treated with 2N HCl (30 min), preincubated (0.2% Triton X-100, 5% NS in PB, 1 hour), and incubated overnight (0.2% Triton X-100, 2% NS in PB, rat anti-BrdU [1:500, Biozol], and rabbit anti-PDGFRα antibodies [1:50, Cell Signaling Technology]) at 4°C for BrdU detection. Three images were taken per coverslip. BrdU−, PDGFRα− and DAPI-positive cells were quantified using the “Analyze particles” tool in Fiji ImageJ. Immunolabeled cells were extracted by specifying suitable size and circularity ranges to optimize cell counts. Percentage of double-positive OPCs from total OPC number was determined.

2.12 | Statistical analysis

Statistical testing was performed using GraphPad Prism 7.0. For all values, mean ± standard error of the mean is given. Shapiro-Wilk normality test was applied for distribution testing. Statistical test is mentioned at the respective position.

3 | RESULTS

3.1 | Myelination pattern and myelin sheath ultrastructure in the dysplastic neocortex

To investigate the extent of myelination in the cortical gray matter of control and FCD IIa specimens, we performed double immunolabeling for nonphosphorylated neurofilament H (SMI32),31 expressed by pyramidal neurons in layers III and V, and CNPase, a marker for myelinated axons.20,33 In controls (n = 12), SMI32 immunolabeling showed bands of pyramidal neurons in layers III and V, with parallel-oriented apical dendrites reaching the lower border of layers I and III,
respectively. Layer IV appeared as a dark, unlabeled gap in-between (Figure 1A). In FCD IIa (n = 8), SMI32-positive pyramidal cells were still arranged in layers, but were less numerous with shortened apical dendrites, in particular in layer V. In addition, strongly SMI32-stained, dysmorphic neurons were present in layers III and V (Figure 1C).

Labeling for CNPase showed that in control tissue a dense, radially aligned network of myelinated fibers extended from the gray-white matter boundary up to layer III, declining gradually from the white matter to the upper layers (Figure 1B). In FCD IIa, a similar pattern of CNPase-immunolabeled fibers was observed, albeit with reduced signal intensity (Figure 1D). Nevertheless, they reached up to layer III as in the nondysplastic neocortex.

Accordingly, when we determined the proportion of the myelinated cortical gray matter, we found no difference in the extent of myelinated areas (approximately 55%-60%) between dysplastic and control cortex (Figure 1E).

Electron microscopy was performed to evaluate the ultrastructure of axons and myelin sheaths in the gray matter of control (Figure 2A) and FCD IIa (Figure 2B) cases. We focused on layers V and VI, since their axons show higher myelin coverage and are more homogeneously myelinated than those in layers II and III.34 The g-ratio, defined as the ratio of the axon diameter to the outer diameter of the myelin sheath, was determined.28

This analysis revealed that the mean axon diameter was almost the same in both groups (controls: 555.7 ± 52.2 nm; FCD IIa: 588.4 ± 66.7 nm; Figure 2C). In addition, the frequency distribution of axon diameters was very similar (controls [n = 4; 183 axons] 209.8-1901.5 nm; FCD IIa [n = 4; 260 axons] 253.8-2173.3 nm) (Figure 2D). The mean g-ratio of myelinated axons, however, was significantly increased in FCD IIa specimens (0.78 ± 0.01) when compared to non-dysplastic controls (0.73 ± 0.007; Figure 2E), indicating that axons in layers V and VI of FCD IIa specimens have a thinner myelin sheath. In addition, plotting all g-ratio values versus all axon diameter values demonstrated that the regression line for FCD IIa axons is increased over the entire axon diameter spectrum when compared to controls (Figure 2F).

### 3.2 Characterization of the oligodendrocyte lineage in FCD

Next, we investigated whether the reduced thickness of myelin sheaths in the dysplastic cortex might be related to a compromised oligodendrocyte lineage. Therefore, we investigated the expression and distribution of PDGFRα, an established OPC marker,20 in FCD specimens in comparison to controls by immunolabeling and real-time RT-qPCR.

In both groups, PDGFRα-positive OPCs were homogeneously distributed across all cortical layers (not shown). The

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**FIGURE 1** Distribution of myelin fibers in the dysplastic and control cortex. Representative photomicrographs of tissue sections (50 µm) from control and focal cortical dysplasia (FCD) IIa cases double immunolabeled for SMI32 (A, C) and CNPase (B, D). In controls and FCD IIa, SMI32-positive pyramidal neurons are densely arranged in layers III and V (A, C), with the appearance of dysmorphic neurons across all layers in the dysplastic cortex (C). CNPase-positive myelin fibers in the control case (B) appear denser than those in FCD IIa (D), but all reach layer III. The border of layer III is marked with a dashed line (A-D). The proportion of the myelinated area in the cortical gray matter is approximately 55%-60% in both groups (E). Ctrl, control. Scale bars: 200 µm
PDGFRα signal was present in the cytoplasm and processes of these cells, reflecting the characteristic morphology of OPCs (Figure 3A) as described before. Cell counting and real-time RT-qPCR revealed that the density of PDGFRα-positive OPCs (controls [n = 11]: 2.74 ± 0.59 cells/mm²; FCD IIa [n = 10]: 1.25 ± 0.26 cells/mm²) (P = .041) was significantly decreased (Figure 3B) and the expression of PDGFRα mRNA was slightly reduced in the dysplastic cortex (controls [n = 14]: 0.12 ± 0.02 rel. exp. mRNA; FCD IIa [n = 10]: 0.08 ± 0.01 rel. exp. mRNA) (P = .082; Figure 3C), suggesting that the OPC pool might be affected in FCD.

To investigate whether the reduced numbers of OPCs might be related to the patients’ history we performed a Pearson’s correlation with clinical parameters of the patient cohort. We conducted correlation analyses and one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test and found that there is no significant correlation between the age at surgery (R = −0.050, P = .673), the epilepsy duration (R = 0.0025, P = .829) or the seizure frequency (P = .803), and the density of PDGFRα-positive cells. Furthermore, it is noticeable that in all correlations, low OPC numbers are mostly attributed to FCD cases (Figure 3D-F).
In addition, we addressed the question whether the observed reduction in PDGFRα-positive OPCs was paralleled by changes in the mature oligodendrocyte population. We performed ISHH for MBP mRNA, which is exclusively expressed in mature, myelinating oligodendrocytes. In controls, MBP mRNA-expressing oligodendrocytes were most numerous in the infragranular layers of the gray matter (Figure 4B,C) and thinned out gradually up to layer I (Figure 4B). In FCD IIa, much fewer oligodendrocytes were detectable; some, located in the deep cortical layers, exhibited very strong MBP mRNA signals (Figure 4E,F). Cell counting revealed reduced numbers in the gray matter of FCD IIa (controls [n = 5]: 190.8 ± 34.7 cells/mm²; FCD IIa [n = 4] 103.7 ± 26.7 cells/mm²) (P = .099; Figure 4G).

3.3 | Proliferation capacity of cultured human OPCs

Finally, we investigated whether the decrease in OPC number might be due to a defect in proliferation. Therefore, we isolated OPCs from control and FCD IIa cortices and determined total numbers of OPCs isolated per gram cortex tissue. In controls, the number of isolated OPCs was negatively correlated with age (r² = .81; P = .01; Figure 5A). In contrast, the total number of isolated OPCs was strongly reduced in FCD IIa when compared to age-matched controls and stayed consistently at a low level, independent of age (Figure 5A). The purified OPCs were cultured for 3 days and proliferation capacity was determined by BrdU incorporation followed by fixation and double immunolabeling for BrdU and PDGFRα (Figure 5F,G). The numbers of PDGFRα- and BrdU-positive OPCs were strongly reduced in FCD IIa tissue when compared to controls younger than 30 years (<30a) (Figure 5B,C) (PDGFRα: control <30a [n = 3], 53.9 ± 12.5 cells/mm²; FCD IIa [n = 4], 11.1 ± 2.0 cells/mm²; BrdU: control [n = 3], 33.6 ± 7.1 cells/mm²; FCD IIa [n = 4], 4.3 ± 1.3 cells/mm²), whereas the number of PDGFRα- and BrdU-positive OPCs from controls >30a was comparable with that of FCD IIa (Figure 5B,C) (PDGFRα: control >30a [n = 3], 10.4 ± 2.9 cells/
When we determined the percentage of BrdU/PDGFRα double-labeled cells, we found that OPCs from FCD IIa tissue incorporated BrdU significantly less than those of control tissue (Figure 5D,E) (control: 57.9 ± 3.6%, FCD IIa: 37.2 ± 6.3%) indicating that the proliferation capacity of OPCs is severely impaired in the dysplastic neocortex. Of interest, although OPC numbers were significantly reduced in controls older than 30 years, they did not show a decreased division rate (Figure 5D) (proliferating OPCs: control <30a [n = 3], 62.7 ± 3.8%; control >30a [n = 3], 55.3 ± 4.4%). In contrast, the proliferation capacity of OPCs was significantly diminished in FCD IIa (Figure 5E) (proliferating OPCs: FCD IIa [n = 4], 37.2 ± 6.3%).

Pearson’s correlation test did not reveal any significant correlation between the percentage of proliferating OPCs and the duration of epilepsy (R = 0.050, P = .89) or seizure frequency (R = 0.240, P = .53) (Figure 5H,I), indicating that the impaired proliferation capacity is not related to epilepsy but rather inherent to the FCD pathology.

4 | DISCUSSION

There is evidence that the FCD pathology is associated with changes in myelination. This has been mostly reported for the cortical white matter. However, we also found alterations in myelination in the dysplastic gray matter. Based on the observation that myelin fibers are distorted and fractured in layer V of the dysplastic neocortex, we investigated in more detail the myelin fiber extension and the ultrastructure of the myelin sheaths.

We found by SMI32/CNPase double immunolabeling that the basic myelination pattern and the proportion of myelinated area are generally preserved. Radially orientated, myelinated fibers extend up to layer III as also shown for FCD IIa by Zucca et al (2016) and in normally developed human cortex. On the ultrastructural level, we focused on layer V, where axons are homogeneously myelinated as shown in the adult mouse neocortex. The relative frequency distribution of axon diameters is basically the same in dysplastic and control cortex. However, myelin sheaths around axons are thinner as proven by an increased g-ratio. We assume that this is attributed to almost all neurons in layer V and not just a feature of the dysmorphic ones, since the increased g-ratio is observed for the majority of measured axons. To specify the affected cell type reconstruction experiments of individual cells will be necessary. Functionally, myelination and axon size are two features that mutually affect each other. On the one hand, larger CNS axons tend to have thicker myelin sheaths, and oligodendrocytes can promote the radial growth of axons by inducing the accumulation of neurofilaments. We did not find any differences in the caliber of the axons between controls and FCD IIa cases, allowing the interpretation that axonal size is not a dominant factor in the reduction of myelination.

An increased g-ratio is typically interpreted as a sign for remyelination and has been extensively investigated in...
the context of MS,37 where OPCs lose their capacity to differentiate into mature oligodendrocytes. Therefore the intrinsic remyelination capacities are impaired.35 In fact, we also found significantly fewer OPCs and a reduction in mature oligodendrocytes in the dysplastic cortex (see below), pointing to a reduced myelination capacity in FCD IIa. We cannot distinguish, however, whether a previous pathological event caused demyelination of axons in the dysplastic area or whether hypomyelination is inherent to the FCD IIa pathology.

One reason for the reduced myelination could be an impaired differentiation of oligodendrocytes. In the adult healthy brain, there is a continuous differentiation of OPCs into myelinating oligodendrocytes and new myelin is permanently generated.21,39 We found that the overall decrease in the myelin content of gray matter axons in FCD IIa is not...
only accompanied by a decrease of mature oligodendrocytes but already by a significant reduction of OPCs. Of interest, also our in vitro proliferation assay demonstrates a reduced proliferation capacity of OPCs isolated from the gray matter, suggesting an early impairment in the oligodendrocyte lineage and a defective expansion of the progenitor pool.

Other previous studies have focused on myelination abnormalities in the white matter, in particular, of FCD IIb cases. Some showed an associated loss of oligodendroglia, whereas others reported that oligodendrocytes and OPCs in the white matter of FCD IIb cases did not differ significantly from that in normal white matter.

Although the reasons for these contradictory findings between our studies and other studies are not clear, they could arise from the fact that we have focused on cortical gray matter. The development of OPCs in disease contexts has been studied extensively in white matter, but much less is known about the dynamics of these progenitors in gray matter regions. To our knowledge, this is the first study to describe alterations in the oligodendrocyte lineage in the cortical gray matter of FCD IIa. Our previous work demonstrated that oligodendrocyte and myelin-associated transcripts are also downregulated in the gray matter of other FCD types (Ia, IIIa) and that myelinated fibers appear fractured and distorted. Future work will clarify whether the myelination defect in the cortical gray matter applies also to other FCD types.

Recent reports have described differences in OPC proliferation and differentiation between gray and white matter. Furthermore, OPCs originating from white or gray matter have different properties and functions. For example, NG2 cells of the white matter have a stronger proliferative capacity than those of the gray matter. Accordingly, slowly proliferating NG2 cells seem to be less efficient in generating mature oligodendrocytes. This could explain why the observed reduction in OPC and oligodendrocyte numbers in the FCD IIa gray matter is more pronounced than described for the white matter.

Of interest, OPCs isolated from controls older than 30 years were also significantly reduced when compared to younger controls. The reduction was comparable with OPCs isolated from FCD IIa specimens. These results are in line with those of previous studies showing that the proportion of OPCs gradually decreases with age in the cortex of NG2-EYFP mice. But unlike in FCD IIa, control OPCs older than 30 years did not show a significant decrease in their ability to proliferate. This result indicates that the observed reduction in OPC number in older controls and FDC IIa is most likely due to different mechanisms and supports the hypothesis that the observed proliferation defect is based on an intrinsic mechanism.

What are the underlying mechanisms for the deficiency in myelination and reduction in oligodendrocyte and OPC cell numbers in FCD IIa? The differentiation of OPCs to myelinating oligodendrocytes is a tightly regulated process depending on intrinsic and extrinsic factors. Our cell culture experiments support the idea that the reduced proliferative capacity of OPCs is intrinsically regulated, since cultured OPCs from FCD IIa specimens show reduced BrdU incorporation in the absence of any cellular environment or neuronal activity. In addition, in our previous study, we found a downregulation of the transcription factor MYRF, which actively regulates the expression of myelin-associated genes and is essential for the termination of OL differentiation.

Finally, the reduced myelin sheath thickness observed in FCD IIa could also result from an impaired myelination process itself affecting the capacity of myelinating oligodendrocytes to wrap the axon with myelin. It has been reported that during myelination, oligodendrocytes wrap the axons with a cytoplasmatic tongue, and during this process, there is a cytoskeletal actin filament turnover that drives the leading-edge growth of the oligodendrocyte during the myelin sheath formation in the CNS. Our microarray analysis displayed a reduction of actin-associated factors in FCD IIIa. We cannot say if this holds true for FCD IIa, but it is possible that the impaired myelination results from deficits of the oligodendrocytes to wrap the axons.

How might an impaired myelination contribute to the epileptogenicity? In vivo experiments in rodents have shown that oligodendrocyte differentiation and myelination occur preferentially in the presence of electrically active neurons, and other studies have shown that ion channel activation or electrical activity regulates myelin formation. Myelin does not only insulate the axons but also induces a clustering of sodium channels at the node of Ranvier, enabling saltatory nerve conduction. The myelin thickness does not only affect conduction velocity but also synchronous firing of action potentials.

A previous study using a cuprizone mouse model has revealed that demyelination can actively contribute to hyperexcitability of gray matter axons by abnormal reorganization of the axolemma, particularly at the nodes of Ranvier. The loss of myelin causes heterogeneous changes such as dispersion of nodal sodium channels into adjacent regions and aberrant organization of anchoring proteins, ultimately resulting in ectopic action potential generation in cortical axons.

In FCD IIa, the underlying pathomechanisms responsible for the intrinsic epileptogenic potential of the dysplastic lesions is not understood. It is possible that the uncontrolled neuronal activity causes an abnormal “crosstalk” between neuronal components and the oligodendroglia, ultimately resulting in oligodendrocyte death and in severe deficits in the axonal ensheathment.

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CONFLICT OF INTEREST
None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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