Pathology of myelinated axons in the PLP-deficient mouse model of spastic paraplegia type 2 revealed by volume imaging using focused ion beam-scanning electron microscopy

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ABSTRACT

Advances in electron microscopy including improved imaging techniques and state-of-the-art detectors facilitate imaging of larger tissue volumes with electron microscopic resolution. In combination with genetic tools for the generation of mouse mutants this allows assessing the three-dimensional (3D) characteristics of pathological features in disease models. Here we revisited the axonal pathology in the central nervous system of a mouse model of spastic paraplegia type 2, the Plp−/− mouse. Although PLP is a bona fide myelin protein, the major hallmark of the disease in both SPG2 patients and mouse models are axonal swellings comprising accumulations of numerous organelles including mitochondria, gradually leading to irreversible axonal loss. To assess the number and morphology of axonal mitochondria and the overall myelin preservation we evaluated two sample preparation techniques, chemical fixation or high-pressure freezing and freeze substitution, with respect to the objective of 3D visualization. Both methods allowed visualizing distribution and morphological details of axonal mitochondria. In Plp−/− mice the number of mitochondria is 2-fold increased along the entire axonal length. Mitochondria are also found in the excessive organelle accumulations within axonal swellings. In addition, organelle accumulations were detected within the myelin sheath and the inner tongue. We find that 3D electron microscopy is required for a comprehensive understanding of the size, content and frequency of axonal swellings, the hallmarks of axonal pathology.

1. Introduction

Focused ion beam-scanning electron microscopy (FIB-SEM) has become one of the highly used methods in the life sciences for the three-dimensional visualization of ultrastructural details in a tissue of interest. These technical developments allowed us to explore complex structures in 3D such as the spindle pole of mouse oocytes (So et al., 2019) or the change of distribution of ribbon synapses at the hearing onset of mice (Michański et al., 2019). Addressing the ultrastructure of glial cells in the nervous system and their interactions with neuronal axons, FIB-SEM has been utilized to study repair mechanisms in peripheral nerves after injury (Gomez-Sanchez et al., 2017), the evolution of ensheathing glia (Weil et al., 2018) and the emergence of pathological outfoldings of myelin in the central nervous system (Erwig et al., 2019).

A variety of protocols exists to visualize structures of interest by serial block face imaging in the scanning electron microscope (SEM) after chemical fixation. For contrasting, the heavy metals osmium tetroxide and uranyl acetate as well as other contrasting agents including thiocarbohydrazide and tannic acid are widely used. Cryo-immobilization followed by freeze substitution has also been used to prepare samples for this imaging mode, aiming at structural preservation as close as possible to native conditions. Both techniques are not without technical challenges and may cause artifacts, necessitating careful interpretation and analysis of data.

In the mammalian nervous system, myelin is among the most difficult to preserve cellular structures, a multilayered sheath comprising lipid-rich membrane bilayers that are spirally wrapped around a central axon (the “axon myelin unit”). Proteolipid protein (PLP), the protein encoded by the PLP1/Plp1 gene, is the major protein of central nervous
system myelin (Jahn et al., 2009; Werner et al., 2013). Indeed, the function of oligodendrocytes in preserving the integrity of myelinated axons (Griffiths et al., 1998; Möbius et al., 2008) involves the oligodendroglial expression of the Plp-gene (Lüders et al., 2017). Point mutations, gene duplications and deletions of the human PLP1 gene give rise to a spectrum of neurodevelopmental disorders (leukodystrophies), ranging from severe Pelizaeus-Merzbacher disease (PMD, most frequently caused by duplication of the PLP1 gene) to the comparably milder spastic paraplegia type 2 (SPG2) (Cailloux et al., 2000; Monasterio-Schrader et al., 2013). Axonal swellings indicate disturbed axonal transport (Edgar et al., 2004) of organelles including mitochondria (Saab et al., 2013; Yin et al., 2016). Therefore, we also revealed details of the content and number of the axonal swellings (also en bloc stained with 2% uranyl acetate overnight at 4 °C) in the PLP-deficient mice (Rosenbluth et al., 2006; Möbius et al., 2016). This structural feature is best preserved by sample preparation applying high-pressure freezing and freeze substitution. We note that most of these pathological characteristics were first observed using traditional two-dimensional transmission electron microscopy (Edgar et al., 2004; Griffiths et al., 1998; Rosenbluth et al., 2006).

Understanding of the SPG2-like disease mechanism leading to axonal pathology in Plp−/Y mice has remained fragmentary. Aiming at improved assessment of the pathological consequences of PLP-deficiency we applied three-dimensional (3D) electron microscopy on optic nerve samples of Plp−/Y mice, which exhibit the same signs of pathology as found in the spinal cord of SPG2 patients. Here, we could reveal details of the content and number of the axonal swellings (also termed spheroids) (Klugmann et al., 1997; Griffiths et al., 1998; de Monasterio-Schrader et al., 2013). Axonal swellings indicate disturbed axonal transport (Edgar et al., 2004) of organelles including mitochondria (Saab et al., 2013; Yin et al., 2016). Therefore, we also addressed morphological alterations affecting axonal mitochondria in Plp−/Y mice. Since mitochondrial morphology is influenced by chemical fixation, we evaluated changes potentially caused by sample preparation, comparing chemically fixed samples with high-pressure frozen and freeze substituted samples. Our data suggest that for volume imaging of mitochondria both methods are suitable. Furthermore we show that 3D analysis of axon-myelin units in Plp−/Y mice reveals important details of the SPG2-like pathology such as differences in the composition of axonal swellings and organelle accumulations within the myelin compartment.

2. Material and methods

2.1. High-pressure freezing and freeze substitution

Plp−/Y mice (Klugmann et al., 1997) kept on a C57Bl/6N background and wild type littermates were sacrificed by cervical dislocation at postnatal day 75 (P75). The optic nerves were dissected, cut and high-pressure frozen (Weil et al., 2019) using 20% Polyvinylpyrrolidone (PVP) as a filler and the HPM100 high-pressure freezer (Leica, Vienna, Austria). An automatic freeze substitution unit (Leica, AF210) was used to perform the freeze substitution. 0.1% tannic acid in acetone was incubated at −90 °C for 100 h followed by three acetone rinses for 30 min each. 2% OsO4, 0.1% uranyl acetate in acetone was used for contrasting the samples (7 h at −90 °C). The temperature was automatically raised to −20 °C within 14 h, kept there for 16 h and raised to 4 °C within 2.5 h. OsO4 was removed by washing four times with acetone for 30 min. The nerves were then rinsed with acetone for 1 h at room temperature. The samples were incubated with increasing concentrations of resin (2:1; 1:1; 1:2) for 2 h each and left in 90% Durcupan over night without component D. The next day the samples were incubated with 100% Durcupan (all components) for 4.5 h and polymerized for 48 h at 60 °C.

2.2. Chemical fixation

Dissected samples were immersed in primary fixative (Karlsson-Schultz phosphate buffer: 109.5 mM NaH2PO4·H2O, 93.75 mM Na2HPO4·2H2O, 86.2 mM NaCl, 2.5% glutaraldehyde, 4% formaldehyde. Adjust the pH to 7.4 and filter) and processed with a modified OTO-protocol (Deerinck et al., 2010), as described previously (Weil et al., 2018). The samples were rinsed three times for 15 min with 0.1 M phosphate buffer at 4 °C. Samples were post-fixed for 3 h with 2% OsO4 and 1.5% K3Fe(CN)6 at 4°C and rinsed three times with H2O for 15 min each at 4 °C. They were further contrasted with 1% thio-carbohydrazide 1 h at room temperature, followed by three times 15 min rinsing with water and 1.5 h incubation with 2% OsO4. After several washes with H2O the samples were en bloc stained with 2% uranyl acetate overnight at 4 °C. The next day the nerve samples were rinsed three times for 15 min with H2O and dehydrated through a series of ascending concentrations of acetone for 15 min each (30%, 50%, 75%, 90%, 3 × 100%). The samples were incubated with increasing concentrations of resin (2:1; 1:1; 1:2) for 2 h each and left in 90% Durcupan over night without component D. The next day the samples were incubated with 100% Durcupan (all components) for 4.5 h and polymerized for 48 h at 60 °C.

2.3. TEM imaging

Ultrathin sections of embedded optic nerve samples were cut using an ultramicrotome (RMC PowerTome PT-PC, Science Services, Munich, Germany) and a 35° diamond knife (Diatome, Biel, Switzerland). Sections were placed on 100 mesh hexagonal copper grids (Science Services) and imaged with a LEO912 electron microscope (Carl Zeiss Microscopy, Oberkochen, Germany) and an on-axis 2 k CCD camera (TRS, Moorenweis, Germany).

2.4. Sample mounting, FIB-SEM and data acquisition

Samples were trimmed using a 90° trimming knife (Diatome) and positioned on a SEM-stub using silver conductive resin (EPO-TEK TEK-129-4). The surface was sputter coated (Leica, ACE 600) with a layer of 10 nm gold and placed inside the FIB-SEM (Crossbeam 540, Zeiss). After exposing a cross-section through the region of interest with 15 nA ion current and polishing with 7 nA, a 400 nm deposition of platinum was done using a 3 nA current. The final dataset was acquired at 1.5 kV (600/1000 pA, ESB grid 450 V) 5 nm × 5 nm × 25 nm voxel size with a milling current of 1.5/3 nA.

2.5. Image analysis

All following steps were performed using the image processing package Fiji (Schindelin et al., 2012): The images were aligned using the SIFT algorithm, cropped and inverted. They were smoothed using a Gaussian blur (sigma 1) and a local contrast enhancement was applied (CLAHE: blocksize 127, histogram bins 256, maximum slope 1.5). The axons and the mitochondria were manually segmented in IMOD (Kremer et al., 1996). The axonal swellings were reconstructed using MIB (Belevich et al., 2016), IMOD and Imaris (Bitplane).
3. Results

3.1. Image quality in high-pressure frozen and chemically fixed samples is sufficient for 3D data acquisition

First we addressed the question whether sample preparation by chemical fixation (CF) or high-pressure freezing (HPF) and freeze substitution (FS) provides samples suitable for volume imaging by FIB-SEM. We found that chemically fixed as well as high-pressure frozen samples exhibit sufficient contrast for FIB-SEM data acquisition (Fig. 1). In the HPF-prepared samples the contrast of microtubules was comparatively weak while in the chemically fixed samples axons were readily recognizable by the pattern of microtubule profiles (Fig. 1A and B). PLP-deficient myelin sheaths are instable and prone to fixation artifacts, probably because PLP is involved in the adhesion of adjacent myelin lamellae (Boison et al., 1995; Klugmann et al., 1997; Rosenbluth et al., 2006). In the datasets of the chemically fixed samples the lamella separation of the myelin sheaths was clearly visible and more pronounced (Fig. 1C), while in the high-pressure frozen dataset the overall appearance of the myelin was more consistent (Fig. 1D). On the other hand the interface between axon and myelin was better preserved in the chemically fixed samples. The occasionally observed separation of the axon and the myelin sheath in high-pressure frozen samples is probably due to distortions during handling of the fresh tissue and freezing (Fig. 1B and D). Taking these respective limitations into account, both sample preparations are useful for 3D data acquisition. Thus, FIB-SEM data stacks are a potential source of information about the shapes of organelles like mitochondria or the size, composition and distribution of organelle accumulations in axonal swellings.

For a careful assessment of the fine structural preservation a higher resolution by transmission electron microscopy was applied and provided more detail at the sub-organelle level, as exemplified by the membranes of axonal mitochondria (Fig. 2, coloured in blue). Mitochondrial cristae structure was differently preserved by the two sample preparation methods. In HPF prepared samples membranes appeared generally smoother. The inner and outer mitochondrial membranes are found in close apposition with an narrow intermembrane space. Also the contrast of cristae and lumen differed in the two sample preparation methods, probably due to unequal heavy metal staining (Fig. 2A and C compared to Fig. 2B and D). To be able to detect possible subtle pathological alterations of axonal mitochondria cristae morphology in Plp−/Y mice high TEM resolution preferentially by EM tomography is required. However, the same samples can be used for TEM, EM tomography and FIB-SEM. We conclude CF provides a better preservation of the axon-myelin interface and more contrast in axonal microtubules while HPF provides better preservation of myelin morphology. The overall mitochondrial structure with cristae is equally well visible in FIB-SEM images, but fine structural aspects of mitochondrial ultrastructure are preferentially studied with high-resolution TEM methods in HPF prepared samples.

3.2. Volumetric analysis reveals an increased number of axonal mitochondria in Plp−/Y mice

To investigate axonal mitochondria, optic nerves from Plp−/Y mice and littermate controls (n = 3) at the age of 75 days were chemically fixed or prepared by HPF and FS (n = 3) and respective 3D datasets covering a volume of about 15 µm × 15 µm × 20 µm (5 nm × 5 nm × 25 nm voxel size) were acquired. Per dataset five myelinated axons were segmented manually using IMOD (Kremer et al., 1996) including axons, myelin and mitochondria (Fig. 3, axonal membrane in yellow, axonal mitochondria in blue). In wildtype mice...
reconstructed axons displayed normally myelinated internodes with an average of 3 mitochondria per 10 µm axonal length (Fig. 3A and B and 4A). In comparison, Plp−/Y mice contained twice as many mitochondria per 10 µm myelinated axon (Fig. 3C and D and 4A). Axonal mitochondria in Plp−/Y mice appear shorter and rounder than those in wt animals, although the individual mitochondrial volume did not significantly differ between both genotypes (Fig. 4B) and, importantly, was not influenced by the sample preparation method (Fig. 4B). Taken together Plp−/Y mice display increased numbers of axonal mitochondria which are not enlarged, but show a different shape.

3.3. A minority of axonal swellings in the Plp−/Y mouse is enriched in mitochondria

Next we determined in FIB-SEM datasets to what extent mitochondrial accumulations contribute to axonal swellings which showed a remarkable diversity in size and content. For example, Fig. 5A illustrates a large axonal swelling containing mainly vesicular organelle accumulations and a few mitochondria. Other axonal swellings in Plp−/Y mice contain a mixture of organelles are smaller (Fig. 5B), and yet other swellings contained largely mitochondrial accumulations (Fig. 5C, Supplementary movie 2). In 8 different FIB-SEM datasets 126 axonal swellings of different sizes (in 70000 µm³ in total) were observed, sometimes in close vicinity to each other (Fig. 6E and Supplementary Table). This averages to one axonal swelling in a volume of 8.2 µm × 8.2 µm × 8.2 µm. From two of the Plp−/Y datasets (high-pressure frozen as well as chemically fixed) four axonal swellings were reconstructed in 3D. Two contained numerous mixed organelles (Fig. 6B/F and Supplementary movie 1), whereas the other two contained exclusively mitochondria (Fig. 6D). One of the reconstructed mitochondrial axonal swellings contained 170 mitochondria within 4.15 µm of axon length (Supplementary movie 2). From the 126 axonal swellings observed in total only 20 (16%) contained mainly mitochondria (Fig. 4C). We note that for a reliable detection of the mitochondrial content of the axonal swelling, the complete organelle accumulation has to be assessed, considering that several axonal swellings showed local heterogeneity within themselves which is typically not seen on thin sections. As an unexpected observation, we also detected organelle accumulations within the inner tongue as well as in myelinic channels of the myelin sheath. Notably, this feature can be easily overlooked and wrongly attributed to the axon. After revisiting the TEM sections, we found an example of accumulation in the inner tongue (Fig. 5D). In the 3D data stack, the localization of such an organelle cluster could be unequivocally assigned to the myelin compartment. In total we found 26 accumulations within the myelin inner tongue or myelinic channels in a volume of 70000 µm³ averaging to one accumulation in a volume of 13.9 µm × 13.9 µm × 13.9 µm (Supplementary Table and Fig. 4C). This finding highlights aspects of myelin pathology contributing to the phenotype of the Plp−/Y mouse.

Another interesting aspect to be addressed by volume EM is the localization of axonal swellings in relation to the myelin sheath and the node of Ranvier. In our datasets we found axonal swellings and organelle accumulations in unmyelinated axonal segments as well as in internodes. Interestingly, one of the reconstructed examples of an axonal swelling occurred in the non-myelinated section of the nodal area. We did not detect a local preference in the occurrence of swellings at specific sites of the axon, e.g. at the paranode. Since the imaged volume did not include complete internodes, a quantitative determination of the swelling distribution along internodes was not possible. Inside the swellings some elements are clearly recognizable as organelles, whereas in other areas the nature of the dense structures is not clearly discernible.

Taken together, 3D FIB-SEM analysis of the axon/myelin-units in Plp−/Y mice is possible on samples prepared by chemical fixation and
OTO-embedding as well as by high-pressure freezing and freeze substitution as long as the general pathology is investigated rather than the fine ultrastructural details of myelin or mitochondria. For the latter EM tomography on HPF-prepared samples is the preferred method. Systematic acquisition and analysis of 3D data stacks provided a first quantitative assessment of the axonal pathology in this mouse model.

4. Discussion

Focused ion beam-scanning electron microscopy (FIB-SEM) allowed us to obtain 3D electron microscopic datasets of mouse optic nerves at high x/y/z resolution. It is a long standing standard that freezing, e.g. by high-pressure freezing, preserves the ultrastructure in a more native and less disturbed state compared to chemical fixation (Vanhecke et al., 2008). Any fine structural analysis therefore aims at imaging vitrified...
samples by cryo-electron microscopy (Koning et al., 2018). HPF and freeze substitution are regarded as the adequate approach for studying subcellular structure–function relationships in 3D (Noske et al., 2008). However, different preparation techniques can be used to prepare plastic-embedded samples for volume imaging by application of FIB-SEM. Depending on the particular question or the local availability of the technique one or the other method might be chosen. At higher resolution, transmission electron microscopy reveals that the membrane preservation is less disturbed after HPF and freeze substitution compared to chemically fixed samples, especially in the case of myelin (Möbus et al., 2010). Both sample preparation techniques may also be used for other 2D and 3D volume imaging techniques. For serial block-face scanning electron microscopy, more focus has previously been put on reducing charging of the sample, either by increasing the heavy metal content in the sample or by charge compensation in the instrument (Deerinck et al., 2018). TEM analyses and 3D electron microscopy by serial block-face imaging in the SEM were already applied for assessment of mitochondrial defects of chemically fixed rat brains (Eustaquio et al., 2018).

In the present study we show that samples prepared for high-resolution TEM analysis by HPF and freeze substitution were also suitable for acquiring FIB-SEM data stacks. Depending on the type of sample, the contrast generated by freeze substitution may not suffice. In such cases, additional contrasting steps may be included in the sample preparation protocol after completion of the freeze substitution (Hall et al., 2018; Steyer et al., 2019). High-pressure freezing combined with freeze substitution is thought to deliver more physiological results than chemical fixation if the tissue preparation is performed properly. This is of particular importance for studies in nervous tissue (Studer et al., 2014). In case of the mouse optic nerve, this white matter tract can be dissected freshly almost intact and fully functional. This tissue is therefore appropriate for preparation by HPF. For studying mitochondria HPF is also applied, since the inner mitochondrial membrane (Höhn et al., 2011) and the distribution of Ca\(^{2+}\) ions is well preserved (Kawahara et al., 2009). We therefore suggest that the findings in HFP-preserved optic nerve reflect the physiological conditions better than the chemically fixed samples and recommend avoiding chemical fixation for fine structural studies in mitochondria.

It is mechanistically not well understood how axonal integrity is impaired by the lack of PLP-expression from myelinating oligodendrocytes. The appearance of axonal swellings (Griffiths et al., 1998) and the increased number of axonal mitochondria (shown here) imply a metabolic imbalance in the axons resulting in a local energetic deficit, at least upon high-frequency axonal activity (Saab et al., 2013). Whether the axonal energy deficit is the direct cause of locally impaired motor driven transport or the cause of abnormal intra-axonal signalling cascades (and thus indirectly for the transport defect of mitochondria and other cargo) remains to be determined. Since we found that axonal swellings can be heterogeneous in composition along their length, large 3D data sets like those provided here are useful to gain insight into the dimensions and content of these organelle accumulations and how they are distributed along the internode.

We note that pathological changes affecting mitochondria were previously also observed by FIB-SEM in CNS axons of mice, in which myelin protein zero (MPZ/P0, the major constituent of peripheral nervous system myelin) was expressed in oligodendrocytes on a PLP-deficient background (Yin et al., 2016). This complex model shows accelerated axonopathy and shorter internodes compared to the Plp\(^{-/-}\) mouse (Yin et al., 2006), probably due to the targeted but aberrant incorporation of P0 into CNS myelin. In this model mitochondrial accumulations were preferentially detected in the juxtaparanodal segment and exhibited a shape change towards a more round morphology with a larger fraction of mitochondria with reduced cristae (Yin et al., 2016). In our SPG2-model we find a similar shape change towards a
more round morphology, but a preferential localization of axonal swellings was not apparent.

Apart from the systematic assessment of the number and composition of axonal swellings and the shape and number of axonal mitochondria, we also found accumulations of organelles within the inner tongue of the myelin sheath. Such pathological features of the axon/myelin units in this mouse model of spastic paraplegia can only be detected and scrutinized by systematic application of 3D imaging by FIB-SEM. Further analysis for example by immunostaining is worthwhile to determine in more detail the content of these axonal swellings. Of special interest to deepen the understanding of the axonal pathology is the fine structure and functional state of the axonal mitochondria inside and outside of the swellings. Such a fine structure analysis requires higher resolution than FIB-SEM and should be performed by EM tomography on HPF-prepared samples.

**CRediT authorship contribution statement**

Anna M. Steyer: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. Torben Ruhwedel: Methodology, Investigation. Christos Nardis: Visualization, Data curation. Hauke B. Werner: Conceptualization, Resources, Writing - review & editing. Klaus-Armin Nave: Funding acquisition, Writing - review & editing. Wiebke Möbius: Supervision, Conceptualization, Funding acquisition, Writing - original draft.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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