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Myelin structure in unfixed, single nerve fibers: Scanning X-ray microdiffraction with a beam size of 200 nm

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ABSTRACT

Previous raster-scanning with a 1 μm X-ray beam of individual, myelinated fibers from glutaraldehyde-fixed rat sciatic nerve revealed a spatially-dependent variation in the diffraction patterns from single fibers. Analysis indicated differences in the myelin periodicity, membrane separations, distribution of proteins, and orientation of membrane lamellae. As chemical fixation is known to produce structural artifacts, we sought to determine in the current study whether the structural heterogeneity is intrinsic to *unfixed* myelin. Using a 200 nm-beam that was about five-fold smaller than before, we raster-scanned individual myelinated fibers from both the peripheral (PNS; mouse and rat sciatic nerves) and central (CNS; rat corpus callosum) nervous systems. As expected, the membrane stacking in the internodal region was nearly parallel to the fiber axis and in the paranodal region it was perpendicular to the axis. A myelin lattice was also frequently observed when the incident beam was injected *en face* to the sheath. Myelin periodicity and diffracted intensity varied with axial position along the fiber, as did the calculated membrane profiles. Raster-scanning with an X-ray beam at sub-micron resolution revealed for the first time that the individual myelin sheaths in unfixed nerve are heterogeneous in both membrane structure and packing.

1. Introduction

This contribution commemorates the occasion of Don Caspar's 90th birthday, celebrates in particular his insightful approach—dating from the late 1960's and extending into the 1980's—to the structure of nerve myelin (Caspar and Kirschner, 1971), and illustrates how his simple directive to one of us (grad student DAK) to "use myelin to learn some X-ray diffraction" developed into nearly 50 years of productive and creative structural biological research on myelin.

The mechanism by which myelin sheaths are formed by the spiral wrapping of oligodendroglial or Schwann cell processes around axons (Bunge, 1968; Geren, 1954; Hirano and Dembitzer, 1967) has recently been further elucidated by three-dimensional (3D) reconstruction from optical and electron microscopic images (Sobottka et al., 2011), by color *Brainbow* (Dumas et al., 2015), and by using embryonic stem cells (Kerman et al., 2015). An alternative model of myelination—involving accretion of "myelinophore organelles"—was based originally on electron microcopy (De Robertis et al., 1958) for CNS, and recently on higher resolution 3D electron tomography for both CNS and PNS (Szuchet et al., 2015). The latter model posits that myelin vesicles (in tubular form) are first synthesized within the cytoplasm of oligodendroglia or Schwann cells and then generate myelin by membrane fusion inside the cell process.

The large number of proteins involved in the elaborate complexity of the "myelinogenic" machinery is underscored by numerous findings: e.g., the formation of myelin basic protein- (MBP) and mRNA-containing granules (Ainger et al., 1997; Carson et al., 1997), the colocalization of MBP and tau and the binding of MBP to microtubules (Dyer and Benjamins, 1989; Karthigasan et al., 1995; LoPresti et al., 1995; Zienowicz et al., 2015), the transportation of RNA granules by microtubules to the myelination site, the role of F-actin in the ensheathment, and the involvement of G-actin and MBP during myelin wrapping (Nawaz et al., 2015; Zuchero et al., 2015). Moreover, the proteins in paranodal and juxtaparanodal areas of the sheath are known to be involved in

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cell-cell adhesion and communication between the neuron and myelin-forming cells (oligodendroglia for CNS, and Schwann cell for PNS) (Arroyo and Scherer, 2000). The axo-glial junction assemblies at the paranode, which have been characterized by electron microscopy (Hirano and Dembitzer, 1982) and electron tomography (Nans et al., 2011; Perkins et al., 2008), consist of a heterogeneous distribution of proteins that are likely associated with lipid rafts (Boggs, 2014), whereas the claudin11-rich interlamellar tight junctions ("radial component") unique to CNS myelin primarily serve to hinder the diffusion of material through the myelin sheath (Denninger et al., 2015).

The principles of colloidal stability and specific protein-protein interactions can account for the extensive membrane-membrane interactions in nerve myelin (Inouye and Kirschner, 2016). The excess positive charge at the cytoplasmic apposition (Inouye and Kirschner, 1988a,b) due to the abundant presence of positively-charged MBPs seems to contradict the closely-apposed membrane surfaces in the internode; however, what may resolve this enigma is the notion that MBP proteins interact via electrostatic and hydrophobic interactions (Min et al., 2009) with specific lipid raft domains of highly negatively-charged PIP2 at the inner leaflet (Zuchero et al., 2015). Intermembrane-MBP phenylalanine-phenylalanine interactions similar to what has been proposed for the intersheet interaction in oligomers of Alzheimer's amyloid beta protein (Aggarwal et al., 2013) could also contribute to close packing at the cytoplasmic apposition. An amyloid-like aggregation of MBP is suggested as a cause for chronic demyelination (Frid et al., 2015). In the compact membrane regions of the myelin sheath, the rafts are likely formed by the interactions between gly cosphingomyelin/cholesterol and P0 or PLP (Hasse et al., 2002), and between MBPs and lipids in the detergent-resistant regions of the membranes (DeBruin et al., 2005).

Novel tools (Simons and Gerl, 2010) have provided accumulating evidence in support of the raft concept as it relates to myelin's spiral wrapping, the adhesion between the nerve and oligodendroglia/Schwann cell, and membrane-membrane interactions in the internode; however, the size and distribution of the rafts have not yet been firmly established (Gandhavadi et al., 2002; McIntosh, 2015). Our previous scanning micro X-ray diffraction (Inouye et al., 2014) directly showed distinctive membrane stackings in glutaraldehyde-fixed single nerve fibers, which is consistent with the preferential association of proteins with specific types of lipid rafts. Because the previous data were collected from fixed PNS rat sciatic nerves and use of a ~1 μm X-ray beam, we sought in the current study to analyze *un*fixed CNS and PNS nerves while using a significantly smaller X-ray beam (~200 nm). We found that the ordered myelin lattices were intermittently distributed, forming micron-sized ordered membrane stackings. Different myelin periods and electron density profiles indicated that these stackings had varied amounts of protein and lipid. Additionally, *en face* scattering from the individual, unfixed myelin sheath gave X-ray reflections arising from distorted membrane lattices, most likely due to Schmidt-Lanterman incisures that form the cytoplasm-containing tunnels connecting the inner and outer loops.

2. Materials and methods

2.1. Animals and sample preparation

Animals were housed at the Biomedical Facility at the European Synchrotron Radiation Facility (ESRF; Grenoble, France), where all procedures were carried out. Optic nerves, spinal cords, corpus callosum, and sciatic nerves were obtained from mature C57BL/6 J or C57BL/6 × 129S3/SvImJ mice (4–12 months of age; obtained from Charles River Laboratories, L'Arbresle, France) and Fischer (F344/IcoCrl) rats (4 months of age; Charles River Laboratories) that had been sacrificed using isoflurane followed by decapitation. All animal procedures were conducted in accordance with protocols complying with federal, state, and local laws and regulations, and approved by the Institutional Animal Care and Use Committees of the respective institutions.

The dissected samples were maintained in Tris-buffered saline (TBS) (5 mM Tris base, 154 mM NaCl, pH 7.4) until subsequent nerve teasing. The preparation of single myelinated fibers from the PNS (sciatic nerve) was carried out as previously described (Inouye et al., 2014), except that the nerves were unfixed and were mounted in specially-designed sample holders (Fig. S1; and see below) rather than sealed in thin-walled, X-ray capillaries. Unlike peripheral (sciatic) nerve, CNS tissue is rather fragile owing to its lack of connective tissue. Therefore, freshly-dissected CNS tissue was first transferred to a standard glass slide (25 × 75 mm), rinsed gently with TBS, and subsequently trimmed with a cleaned scalpel blade to a size of ~2 mm × ~2 mm × 100–150 μm. The slab was then transferred to the surface of a window of silicon nitride (Si_3N_4, henceforth referred to as SiN) (Low Stress Silicon Nitride Window: 2 μm-thick, 5 × 5 mm window; silicon frame, 100 μm thick, 10 × 10 mm; P/N NX10500N_100um; Norcada Inc., 4465 99th Street NW, Edmonton, AB, Canada T6E 5B6, 780-431-9637), and one end was secured with tissue adhesive (Sure + Close II, #SC70307; Medical Solutions International, Inc., 300 Executive Center Drive, Suite 208, Greenville SC 29615-4519, 888-674-4685). Under a dissecting microscope, the slab was teased apart using a stainless steel pin (FST, Inc., item #26002-10) having a tip-size of ~12 μm, yielding 2–3 smaller pieces still connected to one another via either bundles of a few nerves or individual fibers (Fig. S1). The tissue adhesive was then applied to secure the larger pieces onto the surface of the SiN window. A second SiN window was placed over the slab and fibers, additional TBS was added, and the assembly was sealed using vacuum grease (Apiezon N Grease, M&I Materials, Ltd., Ibernia Way, Trefford Park, Manchester, M32 02D UK; +44 (0)161 864 5409) and a UV-activated polymer (Norland Optical Adhesive 68T; P/N 68T30; Norland Products Inc., Cranbury, NJ 08512). The entire teasing process, for both PNS and CNS tissue, was carefully carried out to minimize stretching and damage to the myelinated fibers, which were always in TBS. The entire process, from animal dissection to nerve teasing to sealing the sample holders was approximately 1–1.5 h. Subsequent data collection was carried out over a period of several hours.

Altogether, the X-ray diffraction samples reported here included unfixed rat sciatic nerve, unfixed mouse sciatic nerve, and unfixed rat corpus callosum. In addition, for comparison with previous results that used a 1 μm-beam (Inouye et al., 2014), we prepared fixed rat sciatic nerve treated with 2% paraformaladehyde-2.5% glutaraldehyde (in 0.12 M phosphate-buffered saline, at pH 7.4). We also treated rat sciatic nerve with 10% dimethyl sulfoxide (DMSO) in phosphate buffer—which causes myelin to form, reversibly, a highly-ordered structure with a periodicity about two-thirds that of the native value (Kirschner and Caspar, 1975).

2.2. X-ray diffraction

X-ray micro-diffraction was performed at the ESRF-ID13 beam line. A monochromatic beam of wavelength 0.8319 Å was focused to a spot that was 200 nm × 150 nm (horizontal and vertical directions) full-width at half-maximum (Accardo et al., 2011). Raster-step increments were usually chosen to be larger than the beam size to minimize radiation damage propagating into neighboring scan-points (Accardo et al., 2014). Our previous study of fixed myelinated nerves using scanning microdiffraction with a synchrotron X-ray beam showed that for at least up to 2 s

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