Journal of Structural Biology 190 (2015) 56–72

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

CNS myelin sheath is stochastically built by homotypic fusion of myelin membranes within the bounds of an oligodendrocyte process

Sara Szuchet [a,*], Lauren L. Nielsen [a], Miriam S. Domowicz [b], Jotham R. Austin II [c], Dimitrios L. Arvanitis [d]

[a] Department of Neurology, The University of Chicago, Chicago, IL 60637, USA
[b] Department of Pediatrics, The University of Chicago, Chicago, IL 60637, USA
[c] Advance Electron Microscopy Facility, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637, USA
[d] Department of Anatomy, Histology, Embryology, University of Thessaly, Larissa, Greece

ARTICLE INFO

Article history:
Received 5 December 2014
Received in revised form 25 January 2015
Accepted 27 January 2015
Available online 12 February 2015

Keywords:
Myelinogenesis
Axolemma
Membrane curvature
Glycolipid tubulation
Oligodendrocyte plasma membrane
Multiple sclerosis

ABSTRACT

Myelin – the multilayer membrane that envelops axons – is a facilitator of rapid nerve conduction. Oligodendrocytes form CNS myelin; the prevailing hypothesis being that they do it by extending a process that circumnavigates the axon. It is pertinent to ask how myelin is built because oligodendrocyte plasma membrane and myelin are compositionally different. To this end, we examined oligodendrocyte cultures and embryonic avian optic nerves by electron microscopy, immuno-electron microscopy and three-dimensional electron tomography. The results support three novel concepts. Myelin membranes are synthesized as tubules and packaged into "myelinophore organelles" in the oligodendrocyte perikaryon. Myelin membranes are matured in and transported by myelinophore organelles within an oligodendrocyte process. The myelin sheath is generated by myelin membrane fusion inside an oligodendrocyte process. These findings abrogate the dogma of myelin resulting from a wrapping motion of an oligodendrocyte process and open up new avenues in the quest for understanding myelination in health and disease.

1. Introduction

The term "myelins" defines a broad class of structures consisting of multilamellar tubes composed of nested cylindrical bilayer membranes (Reissig et al., 2010). In the biological context, myelin is a multilayer membrane – synthesized in the central nervous system (CNS) by oligodendrocytes (OLGs) – that envelops axons, and whose best characterized function is that of a facilitator of rapid nerve conduction. By covering their axons with a myelin sheath, vertebrates saved energy, space and developed a highly efficient mode of communication, but they also became susceptible to a large number of diseases affecting myelin – the most prevalent being multiple sclerosis (rev. by (Lassmann, 1998)).

Advances in electron microscopy and low-angle X-ray diffraction spearheaded research on the ultrastructure of myelin. It began with the peripheral nervous system (PNS) because of the unambiguous identification of the Schwann cell as the myelinating cell. By the time researchers turned their attention to CNS myelin, the ultrastructure of PNS myelin had already been defined. It is pertinent to reflect on the ideas and experimental evidence that gave birth to the current hypothesis on myelin sheath formation and on its staying power despite fundamental flaws. The large body of work on this topic has, insightfully, been reviewed by Bunge (Bunge, 1968), who grouped the prevailing ideas into three concepts. *Concept 1* (Luse, 1956) – derived from studies of mouse and rat brains and spinal cord – sustained that CNS myelin originates from a patchwork assembly of many different glial processes arising from one or more glial cells. *Concept 2* referred to the daring hypothesis – originated from work on kitten brain and spinal cord – that the myelin sheath was formed by the coalescence of intracytoplasmic membranes rather than from the plasma membrane (De Robertis et al., 1958). Significantly, as pointed out by Bunge (1968): "this concept had no subsequent proponents". *Concept 3* – supported by many laboratories (Maturana, 1960; Peters, 1960a,b) – held that a glial cell process was applied onto an axon and wrapped around it; after extruding its cytoplasmic

Abbreviations: Abs, antibodies; CNS, central nervous system; GC, galactocerebroside; MBP, myelin basic protein; MyMs, myelin membranes; MFOs, myelinophore organelles; OLGs, oligodendrocytes; PLP, proteolipid protein; PNS, peripheral nervous system; TEM, transmission electron microscopy; 3D-ET, three-dimensional tomography.

* Corresponding author at: 5841 S. Maryland Ave, The University of Chicago, Department of Neurology MC2030, Chicago, IL 60637, USA. Fax: +1 773 702 4066.
E-mail address: szuchet@neurology.bsd.uchicago.edu (S. Szuchet).

http://dx.doi.org/10.1016/j.jsb.2015.01.015
1047-8477/

content, the plasma membrane became oriented into a compact spiral. *Concept 3* faired best mainly because most of subsequent studies were done on mature CNS myelin, whose ultrastructure closely resembles its PNS counterpart. It was, therefore, assumed that the two sheaths originated in a similar fashion. A graphic visualization of *Concept 3* was advanced by Hirano and Dembitzer (1967); they depicted an unrolled myelin sheath as shovel-shaped and surrounded by a continuous thickened rim of cytoplasm. *Ipso facto* – and despite being seriously contested (Knobler et al., 1974, 1976; Lampert, 1965) – this model became the dogma. And, henceforth, has populated every textbook, review, and article written about myelin.

The original wrapping hypothesis (carpet crawler) has also been contested by new models that evolved from observations resulting from the use of advanced imaging technologies. Thus, the finding that an association between an axolemma protein (caspr) and an OLG plasma membrane protein (neurofascin-155) persists throughout myelination, prompted Pedraza et al. (2009) to argue about the inconsistency of the advancement of the inner mesaxon. They proposed in lieu what is referred as the Serpent or Yo–Yo model. Another model – Liquid Croissant – deduced from high-resolution confocal live imaging of living murine organotypic cerebellar slice cultures (Sobottka et al., 2011) states that myelin formation occurs by "pouring out" myelin into a triangular shaped OLG process and myelin thickening is achieved by new layers forming on top of the inner one. Yet another model – Corkscrew – pictures an OLG process wrapping in a corkscrew-like spiral around the axon followed by a spreading of the membrane to form short cuffs of glial cytoplasm (Ioannidou et al., 2012). The common denominator to these models is that they exclude wrapping by the so-called inner tongue. Surprisingly, Snaidero et al. (2014) suggest that myelin grows by the wrapping of the leading edge at the inner tongue. Implicit in all these models is the notion that the myelin sheath is no more than superposed layers of a chemically modified and flattened OLG process. What is in defiance is the manner in which the multilayer structure is assembled.

Given that OLG plasma membrane and myelin are compositionally distinct beyond the major proteins (Jahn et al., 2009; Szuchet et al., 1988; Vanrobaeys et al., 2005), questions concerning myelin synthesis, transport and deposition are highly pertinent and, yet, remain largely unanswered. We have addressed these topics using transmission electron microscopy (TEM), immuno-EM and three-dimensional electron tomography (3D-ET) on an *in vitro* model consisting of pure cultures of OLGs and on embryonic (E) avian optic nerve at stages E12, E15, E17 and E21. The results lead us to set forth three novel and cardinal concepts concerning myelin membrane synthesis, transport, maturation and sheath formation. As a corollary to these concepts, we draw a distinction between "myelin membrane" and "myelin sheath".

2. Materials and methods

2.1. Culture of ovine oligodendrocytes

OLGs were isolated from 3 to 6 months old lamb brains according to our standard procedure (Szuchet et al., 1980). On the average, 2×10^8 cells are obtained. Cells were maintained in culture as described by Szuchet and Yim (1984). Freshly isolated OLGs were plated on tissue culture petri dishes, where they do not adhere but form floating clusters. After 3–5 days, non-adherent OLGs were harvested, centrifuged, resuspended in medium (DMEM plus 20% horse serum, 2 mM L-glutamine and antibiotics) and seeded on polylysine-coated petri dishes; cultures were kept at 37 °C. Alternatively, OLGs were adhered on plates coated with GRASP after pre-coating with polylysine (Schirmer et al., 1994) and maintained in a synthetic medium without serum.

2.2. Avian optic nerve preparation

Fertilized White Leghorn chicken eggs were incubated at 37.9 °C at 60% humidity in a Midwest incubator with automatic egg turning, regularly hatching at day-22 of incubation.

Avian embryos at the different indicated ages were perfused with 4% paraformaldehyde in phosphate buffered saline (PBS), the optic nerves and chiasm were dissected out, placed into planchettes containing 0.35 M Sucrose and frozen via a high-pressure freezer (Baltec HPM 010). The tissue was freeze substituted (Leica AFS2 system) in 0.25% glutaraldehyde (Electron Microscopy Sciences 16530), 0.1% uranyl acetate (Electron Microscopy Sciences 22400) in acetone (Electron Microscopy Sciences 10015) and embedded in lowicryl HM20 resin (Electron Microscopy Sciences 14345).

2.3. Immunohistochemistry

Antibodies: The GC antibody used in Fig. 3A is a commercial antibody that the vendor (Millipore Corporation, CA) claims recognizes the avian glycolipid. We have tested this Ab to confirm its specificity prior to using it. The MBP antibody is anti-rat; it is a generous gift from the late Dr. David Colman (Colman et al., 1982). We have used this antibody extensively in the laboratory to stain ovine, human, avian tissues and cultured OLGs. It is an excellent antibody; it works at concentrations of 1:4000 for Westerns and 1:1000 for immunohistochemistry (Dhaunchak et al., 2010; Fex Svenningsen et al., 2003; Pedraza, 1997). The AA3 anti-PLP/DM-20 monoclonal antibody recognizes epitope 264–276 (FAVLKLMGRGTKF-COOH) of bovine PLP (Yamamura et al., 1991). It has been widely employed; it was shown by others to recognize avian tissue (Perez Villegas et al., 1999). We have used a supernatant (a kind gift from Dr. Wendy Macklin, University of Colorado at Denver); it recognized specifically avian PLP.

Thin sections (80–150 nm) were cut on an ultramicrotome (Leica UC6), placed onto formvar coated gold grids (Electron Microscopy Sciences FCF2010-AU) and single immunolabeled. For GC immuno-staining, sections were incubated with 0.1 M HCl for 10 min, washed with water, blocked with 5% normal goat serum (NGS) in PBS for 2 h, incubated with 1:400 GC monoclonal antibody (MAB342; Millipore, CA) in 2% NGS for 2.5 h, washed with PBS-0.5% Tween 20 (PBST), incubated with 1:100 goat anti-mouse IgG conjugated to 15 nm gold particles (Ted Pella 15752) in 2% NGS for 1 h, washed with PBST and washed with water. For MBP immuno-staining, sections were incubated with 0.1 M HCl for 10 min, washed with water, blocked with 5% NGS for 1 h, incubated with 1:300 polyclonal anti-bovine MBP antibody in 2% NGS for 3 h, washed with PBST, incubated with 1:20 goat anti-rabbit IgG conjugated to 15 nm gold particles (Ted Pella 15727) in 2% NGS for 1 h, washed with PBST and washed with water. For PLP immuno-staining, sections were incubated with 0.2% Triton X-100 (Sigma T-9284) in PBS for 10 min, washed with water, blocked with 2% NGS for 1 h, incubated with 1:2 anti-PLP rat monoclonal IgG (supernatant from clone AA3) in 1% NGS for 3 days, washed with PBST, incubated with 1:30 goat anti-rat IgG conjugated to 15 nm gold particles (Ted Pella 15767) in 1% NGS for 1 h, washed with PBST and washed with water. Sections were stained with uranyl acetate and lead citrate (Electron Microscopy Sciences 17800) to increase the membrane contrast. Images were collected on an electron microscope (FEI Tecnai TF30) operating at 300 kV at varying magnifications.

2.4. Serial section 3-D tomogram collection and modeling

Thick sections (150–300 nm) were obtained on an ultramicrotome (Leica UC6), transferred onto formvar coated copper grids