Experimental Report

Proposal: **8-02-663** **Council:** 10/2012

Title: Neutron Diffraction from Myelin: Novel Differences Between PNS and CNS Membrane Multilayers

This proposal is continuation of: 8-02-594

Researh Area: Biology

Main proposer: **KIRSCHNER Dan**

Experimental Team: KIRSCHNER Dan
DENNINGER Andrew

Local Contact: DEME Bruno
CRISTIGLIO Viviana

Samples: sciatic & optic nerves, spinal cords, from mice, unfixed, in defined D2O-saline, pH~7

Instrument	Req. Days	All. Days	From	To
D16	4	4	09/07/2013	13/07/2013

Abstract:

Rapid nerve conduction in vertebrates depends on myelin's ensheathment of axons. Conduction abnormalities and severe debilitation is due to de-, dys-, and a- myelination. Electron microscopy can provide an essential description of myelin morphology, but compared to diffraction lacks the requisite resolution, sampling, and non-invasiveness to delineate molecular organization and dynamic interactions. About 40 years ago, we showed the complementarity of neutron diffraction (ND) to x-ray diffraction (XRD) for myelin structural studies. At the ILL, we have now demonstrated the vast improvement in temporal and spatial resolution of current instrumentation, recorded the first ND patterns from CNS myelin, tested a flow cell for studying in real-time D-H exchange in myelinated nerves, detailed the difference in diffusion barriers in PNS versus CNS myelinated nerves, and found that specialized junctions unique to CNS myelin modulate diffusion in myelin. We now propose to explore the application of alternative sources of contrast within myelin by in vivo incorporation of deuterium into membrane components, and by incubation or mediated membrane insertion of deuterium-labelled compounds.

ILL Experiment No: 8-02-663
Title: Neutron Diffraction from Myelin:
Novel Differences between PNS and CNS Membrane Multilayers
Experimental Team: Kirschner, Denninger, Feller, Deme, Cristiglio, Zaccai
Local Contacts: Deme, Cristiglio, LeDuc

Background:
Rapid nerve conduction in vertebrates depends on the ensheathment of its axons with myelin. Because alterations in normal myelin structure and function can result in conduction abnormalities and severe debilitation, the structure of myelin has been studied in the native state, from animal models of human myelinopathies, and in tissue that has been physically or chemically treated to induce structural changes. X-ray diffraction (XRD) has been used extensively in the study of myelin structure, and neutron diffraction (ND) can offer complementary information to XRD regarding the distribution of lipid, protein, and water in the sample. Additionally, it may be used to measure water diffusion kinetics in myelin by real-time exchange with heavy water (D_2O), which may reveal more subtle defects in myelin physiology. Despite promising results provided by ND from rabbit sciatic nerves (peripheral nervous system; PNS) >40 years ago (Kirschner et al., 1975), no substantive follow-up experiments were undertaken. Moreover, it was unclear if useful information could be obtained from much smaller mouse PNS samples or from even smaller mouse central nervous system (CNS) optic nerves, for which numerous transgenic models of human disease have been developed. To revisit ND as a potential tool for structural biological characterization of myelin, we undertook new experiments at the ILL from November 2-4, 2010 (TEST-1875), and successfully collected data from rat sciatic (PNS) and optic (CNS) nerves. We next extended our analysis to include mouse nerves (Exp. No. 8-02-570), and most recently, we established differences in D_2O-H_2O exchange kinetics in a mouse lacking a CNS tight junction protein (Exp. No. 8-02-594).

Objectives:
Previous experiments relied entirely upon a simple bulk exchange with D_2O to provide contrast to the tissue. This simple exchange confines the deuterium to aqueous spaces and molecules with readily-ionizable chemical groups. Although useful, this type of exchange limits the types of questions that can be addressed using ND. Addressing novel questions of myelin biology requires identifying alternative sources of contrast. Our objectives for the experimental period reported here included: (1) the in vivo incorporation of deuterium into membrane components (lipids, carbohydrates and proteins) through administration of non-toxic quantities of D_2O to mice via their drinking water; (2) in vivo incorporation of deuterated mevalonate into CNS myelin cholesterol through intracisternal injection; and (3) in vitro labeling of myelin with deuterated non-water small molecules (e.g., hexane, butanol).

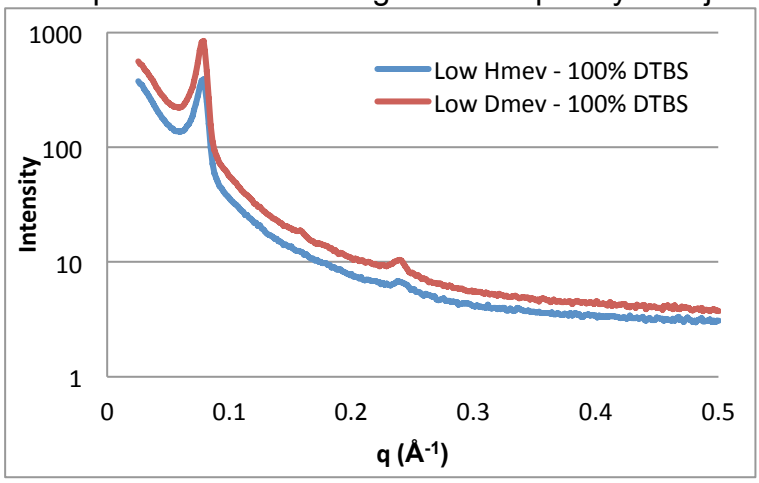
Materials & Methods:
Briefly, sciatic nerves and spinal cords were dissected from mature Fischer rats or C57Bl/6 mice, tied off with silk suture, and maintained in buffered saline (154 mM NaCl, neutral pH) of varying D_2O:H_2O ratios. Samples were loaded into quartz capillary tubes containing buffer with a known D_2O:H_2O ratio and sealed. Exposure times were typically between 1-2 h. For experiments involving in vivo and in vitro labeling of myelin, details of the treatments will be given alongside the relevant data in the Results section below.

Results:
Objective 1 – D_2O feeding
To broadly label myelin membrane components with deuterium, we planned to feed D_2O to mice during development via their drinking water at varying levels (0%, 15%, and 30% D_2O) for several months. Administration of D_2O in this manner has been shown to be a simple

way of labeling myelin lipids and proteins (Ando et al., 2003). After receiving approval to carry out these experiments from the ILL and ESRF (where the animals would be housed), we were informed by the ESRF safety group that the experiments could not be performed because of the poor containment of deuterium in such experiments. After several months of delays, the experiments were cancelled by the ESRF. Because of the time involved in making arrangements at another facility, applying for animal protocols, and the treatments themselves, we were left with insufficient time to carry out these experiments.

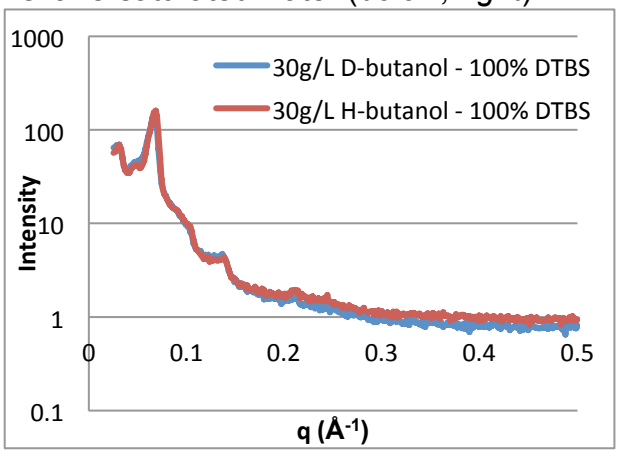
Objective 2 – Intracisternal injection of H/D-mevalonate

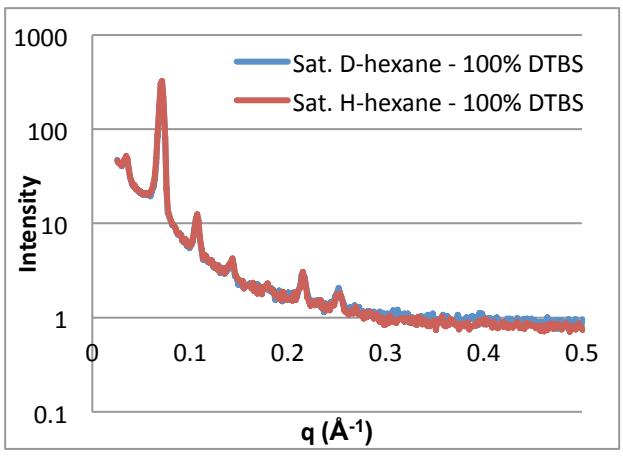
To specifically label a single membrane component, we planned to administer H- and D-mevalonate at varying levels to rats via injection through an intracisternal cannula over several months. Mevalonate is a committed cholesterol precursor, and administration of labeled mevalonate results in strong labeling of CNS myelin cholesterol (Chevallier & Gautheron, 1969). Again, experiments were approved but delayed by the ESRF safety group because of the traceability of the deuterium. After several months of delays, the experiments were eventually approved. However, we were left with approximately 1 week to complete the protocol, so to salvage the experiments the dosage and frequency of injections were increased significantly. Unfortunately, the increased dosage was harmful—rats receiving high doses either died during treatment or experienced seizures. The treatments were suspended after 2 doses, which would provide an insufficient amount of mevalonate to result in any significant labeling of cholesterol. As expected, neutron diffraction patterns from low-dose H/D-mevalonate-treated rat spinal cords (*right*) showed no significant differences.



Objective 3 – In vitro labeling of myelin with H/D-hexane and H/D-butanol

To test whether neutron diffraction could be used to determine the localization of non-water deuterated molecules in myelin, we treated nerves overnight with H/D-hexane or H/D-butanol and analyzed them using neutron diffraction. We carried out experiments at various concentrations of these molecules, but found no significant differences between the samples treated with H and D forms of the molecule. Representative diffraction patterns are displayed for rat sciatic nerves treated with 30g/L H/D-butanol (*below, left*) and H/D-hexane-saturated water (*below, right*).





Conclusions:
Objective 1 planned to use a well-characterized method for labeling myelin and had the highest potential for success. This objective was not completed because of bureaucratic, non-experimental issues. *Objective 2* involved a more risky method for labeling myelin and was carried out under non-ideal circumstances because of bureaucratic, non-experimental issues. The lack of labeling observed in these experiments is most likely a result of the limited timeframe allowed for labeling—days rather than months. The observed effects of high doses of mevalonate on the animals will be considered when planning future experiments and may be used as an upper limit for future treatments. *Objective 3* was the most risky in that these types of treatments are not frequently performed on myelin and are potentially damaging to biological membranes. Unfortunately, these experiments became the focus of the experimental period because of difficulties performing the other two objectives. Whereas the results for this 3rd objective are inconclusive, they do demonstrate the difficulty in performing this type of experiment with live tissue, rather than artificial membranes.

Future Experiments:
Our future work will focus on performing the experiments originally planned in our proposal, with minor modifications. We intend to perform labeling experiments at Boston College (BC) Animal Care Facility, where administration of D_2O to animals has been approved. Animals will be treated at BC, and samples will be obtained, aldehyde-fixed, and transported to the ILL for analysis, thus avoiding the issue of D_2O-containment. We also intend to perform cholesterol-labeling experiments using either mevalonate as described above or deuterated cholesterol (Scott et al., 1980). Again, all samples will be prepared in advance and transported to the ILL for analysis. For Objective 3, more XRD diffraction experiments would be necessary in order to determine an effective way of labeling the myelin without inducing harmful structural changes.

References:
Kirschner, et al. (1975) Neutron diffraction of nerve myelin. In: Neutron Scattering for the Analysis of Biological Structure (Ed. BP Schoenborn), Brookhaven Symposium in Biology, No. 27, III, 68-76.

Ando S, Tanaka Y, Toyoda Y, & Kon K (2003) Turnover of myelin lipids in aging brain. Neurochem Res 28(1): 5-13.

Chevallier F & Gautheron C (1969) A method for the study of cholesterol biosynthesis in the central nervous system. incorporation of [2-^{14}C]mevalonic lactone after intraperitoneal, intracisternal and intraventricular administration in the rat. J Neurochem 16(3): 323-331.

Scott SC, Bruckdorfer KR, & Worcester DL (1980) The symmetrical distribution of cholesterol across the myelin membrane bilayer determined by deuterium labelling in vivo and neutron diffraction. Biochem Soc Trans 8(6): 717.