**Dec 17, 2021**

**Title:** Metabolically-Incorporated Deuterium in Myelin Localized by Neutron Diffraction and Identified by Mass Spectrometry

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**Keywords:**

Myelin / Deuterium / Neutron diffraction / PNS / CNS / Membrane structure / Per-deuteration / Metabolic isotope labeling / Lipids / Lipidomics / Sciatic nerve / Trigeminal nerve / Mass spectrometry / Mice /

**Abbreviations:**

$A_i$, abundance of isotope; CNS, central nervous system; DG, diacylglycerol; FA, fatty acid; HexCer, cerebroside; $M_i$, mass of monoisotope ($i$ = 0) and of the isotopologues ($i$ > 0); MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PNS, peripheral nervous system; PS, phosphatidylserine;, SHexCer, sulfatide; SM, sphingomyelin; TG, triacylglycerol.

We dedicate this article to the memory of Dr. Isabelle Grillo (1973-2019), our colleague at the Institut Laue-Langevin.

**Abstract**

Myelin is a natural and dynamic multilamellar membrane structure that continues to be of significant biological and neurological interest, especially its biosynthesis and assembly in the context of its normal formation and renewal, and pathological breakdown. To explore further the usefulness of neutron diffraction in the structural analysis of myelin, we investigated the use of in vivo labeling by metabolically incorporating via drinking water non-toxic levels of deuterium ($^2$H; D) into pregnant dams and their developing embryos. All of the mice were sacrificed when the pups were about 60 days old. Myelinated nerves were dissected, fixed in glutaraldehyde and examined by neutron diffraction. Parallel samples that were unfixed were frozen for mass spectrometry (MS). Analysis of the neutron diffraction patterns of the sciatic nerves from deuterium-fed mice (D-mice) versus the controls (H-mice) showed no appreciable differences in myelin periodicity, but major differences in the intensities of the Bragg peaks. The neutron scattering density profiles showed an appreciable increase in density at the center of the membrane bilayer in the D-mice, particularly in the pups. MS analysis of the lipids isolated from the trigeminal nerves demonstrated that the level of D was greater in the pups compared to their mother—97.6% ± 2.0% (n=54; range 89.6%–99.6%) versus 60.6% ± 26.4% (n=27; range 11.4%–97.3%). Deuteration in the mother also varied by lipid species, and among lipid subspecies. Three molecular species of phosphatidylcholine (PC), phosphatidylinositol (PI), and diglycerol (DG) were the most deuterated lipids, and sulfatide (SHexCer), one species of sphingomyelin (SM), and triacylglycerol (TG) were the least deuterated. The distribution pattern of deuterium in the D-pups was always bell-shaped, and the average number of D atoms ranged from a low of ~4 in fatty acid (FA), to a high of ~9 in cerebroside (HexCer). By contrast, in D-dam only about one-third of the lipids had symmetric bell-shaped distributions; most had more complex, overlapping distributions that were weighted toward a lower average number of D atom labels. The average number of D atoms ranged from a low of ~3–4 in FA and in one species of SHexCer, to a high of 6–7 in HexCer and SM. In D-pups, the consistently high level of deuteration can be attributed to their *de novo* lipogenesis during gestation and continuation of rapid myelination postnatally. In D-dam, the widely varying levels of deuteration of lipids likely depends on the relative metabolic stability of the particular lipid species during myelin maintenance in the mature animal. Our current findings demonstrate that stably-incorporated D label can be detected and localized using neutron diffraction in a complex tissue such as myelin; and moreover, that MS can be used to screen a broad range of deuterated lipid species to monitor differential rates of lipid turnover. In addition to helping to develop a comprehensive understanding of the *de novo* synthesis and turnover of specific lipids in normal and abnormal myelin, our results also suggest application to myelin's proteins, as well as more broadly to the molecular constituents of other biological tissues.

## Introduction

Diffraction studies of myelin membranes and their molecular constituents have helped to characterize the molecular conformations and organization that underlie myelin stability and instability (Inouye et al., 2017, and references therein). These issues are important because of myelin's essential role in fast, efficient nerve conduction and involvement in a variety of neurological conditions caused by or resulting in demyelination (Lazzarini et al., 2004). Because myelin is a multilamellar stack of membranes, lamellar diffraction has been especially useful in providing information about the general distribution of lipids, protein, and water across the membranes (Kirschner & Blaurock, 1992) and also about the localization of specific lipids (e.g., cholesterol; Caspar & Kirschner, 1971; phosphatidylethanolamine plasmalogen; Kirschner et al., 1982) and proteins (e.g., myelin basic protein, P0 glycoprotein; Kirschner & Ganser, 1980). The majority of diffraction studies of myelin use x-rays; however, neutron diffraction offers a particular advantage owing to the large difference in scattering lengths between hydrogen (H) and deuterium ($^2$H or D), and hence between $H_2O$ and $D_2O$ (deuterium oxide; heavy water). This difference suggests the use of both deuterium exchange and deuterium labeling. With the former, simple diffusion can result in a stable change in the relative amounts of $H_2O$ and $D_2O$ in the aqueous compartments of multilamellar myelin, including membrane regions that have accessible, exchangeable hydrogens. Thus, the distribution of water across the membrane bilayer as well as the kinetics of $H_2O/D_2O$ exchange can be directly determined using these isotopologues of water (Kirschner et al., 1975; Denninger et al., 2014) that cannot be distinguished using x-rays. Neutron diffraction studies using H-D exchange have confirmed the thickness of the water-excluding hydrocarbon domain deduced from x-ray diffraction (Blaurock, 1971; Caspar & Kirschner, 1971), and the rate of D-H exchange in the water-accessible spaces (Denninger et al., 2015). Deuterium labeling that is non-covalent can be achieved by diffusing in a deuterated compound to localize the moiety, e.g., deuterated DMSO (Kirschner, 1974); whereas deuterium labeling via covalent bonding is achieved either by the introduction of stable deuterated precursors of myelin's biochemical constituents (e.g., D-cholesterol, Scott et al., 1980; D-choline, D.L. Worcester, personal communication, 2019), or by giving $D_2O$-spiked drinking water to laboratory animals (Ando et al., 2003; Ando, Tanaka 2005). In the case of metabolic precursors, the labeled compound must first pass through either the blood-nerve barrier (for PNS myelin) or the blood-brain barrier (for CNS myelin) before it can target a particular molecule. By contrast, introducing deuterium via $D_2O$-spiked drinking water ensures that the label is incorporated metabolically into the full complement of the body's molecular constituents that comprise its cells and tissues.

Application of stable isotope-labeling and mass spectrometry (MS) to studying lipid turnover in mouse brain myelin was first undertaken by Ando et al. (2003, 2005). In this pioneering work, 30% $D_2O$ in drinking water was given for 4–6 weeks to mice aged from five to 106 weeks of age, their cerebellar myelin was isolated, and the lipids extracted therefrom. The differential incorporation of deuterium and their disappearance were monitored by MS. The mice in the youngest group were developmentally just beyond their most rapid accretion of myelin and thus were drinking the $D_2O$-spiked water during a relatively slower rate of myelinogenesis (Agrawal et al., 2009). Mice in the older groups were all in myelination periods of either slow accretion or “steady-state” maintenance of the myelin sheath.

In the current study, we administered $D_2O$ to pregnant mice via their drinking water, and continued this protocol for both the dam and pups for two months postpartum. Neutron diffraction was used to detect the structural localization of deuterium after its metabolic incorporation into myelin, and MS to identify the lipid species containing this label. We observed clear, measurable differences in the neutron diffraction patterns obtained from sciatic nerves and spinal cords between $H_2O$ and $D_2O$-fed dams (H-dam, D-dam) and their pups (H-pup, D-pup). In the current report, we focus on results and analysis of the tissue from the PNS. Neutron scattering length density profiles of the sciatic nerve myelin membranes showed a prominent increase in scattering density in the middle of the membrane bilayer. Using mass spectrometry, we confirmed the presence of deuterium-labeled lipids—PE, PC, PS, HexCer, SHexCer, SM, PI, TG, DG, FA—in the myelinated tissue (trigeminal nerve) from the $D_2O$-fed mice. Whereas the lipids from the D-pups averaged ~98 ± 2% deuterated, the lipids from the D-dam were variably deuterated, averaging ~61 ± 26%, which depended on lipid species and subspecies. The consistent, uniformly high incorporation of D into lipids for the D-pups vs. that for D-dam can be explained by the de novo biosynthesis and myelin assembly in the former vs. differential turnover of lipids in mature myelin. In summary, we demonstrated that neutron diffraction together with mass spectrometry can be used to detect and identify deuterium that is stably-incorporated via normal metabolism into the rapidly- and newly-synthesized myelin as well as into myelin undergoing metabolic maintenance where lipid turnover is more targeted.

## Materials & Methods

*Reagents and Standards*

Methyl-tert-butyl-ether (MTBE) and chloroform were purchased from Sigma Aldrich; methanol was from Fisher Science Education; ammonium acetate was from Fluka; and $D_2O$ was from Sigma-Aldrich Corp. (Boston College: 99.9% D, and from Eurisotop, St-Aubin, FR (ILL: 99.9% D).

*Specimens*

All animal procedures were conducted at the Boston College Animal Care Facility in accordance with protocols approved by the Institutional Animal Care and Use Committee at Boston College. Three timed-pregnant C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA), housed on a 12-hour light/dark cycle, and provided *ad libitum* access to food and water. At gestational day 12, when the dams were ~10 weeks old, the mice were separated into treatment groups, in which their drinking water was replaced with water containing 15% (D-mice) or 0% (control, H-mice) $D_2O$. Mice were maintained as such through birth and until postnatal day 7, at which point the volume fraction of $D_2O$ in their drinking water was increased to 20% (D-mice). At postnatal day 13, their drinking water was again increased to 30% volume fraction of $D_2O$. Pups were weaned at postnatal day 28, and all mice were maintained on 30% $D_2O$ (D-mice) or 0% $D_2O$ (control, H-mice) until the pups reached postnatal day 55, at which point all mice (including the dams at 131 do) were sacrificed (see Suppl. Fig. S1) and tissues were harvested as described in the following. The chosen level of $D_2O$ in drinking water was below the threshold for toxicity for mice (Koletzko et al., 1997; Katz et al., 1962; Ando et al., 2003).

*Sample Preparation*

Mice were sacrificed by $CO_2$ asphyxiation, followed by decapitation. Brains (including optic nerves) and trigeminal nerves (which, like sciatic nerves, are peripheral nerves) were removed, flash frozen, and stored at -80°C until further prepared for mass spectrometry. Sciatic nerves and sagittally-bisected segments of spinal cord were removed for analysis by neutron diffraction. Sciatic nerves were tied off at both ends with silk suture and placed under gentle tension on plastic sample holders, while spinal cord segments were laid flat, adhering naturally to filter paper. These samples were immersion-fixed in 1% paraformaldehyde and 1.5% glutaraldehyde in 0.12 M phosphate buffer pH 7.4 overnight at room temperature. Fixed samples were then equilibrated against phosphate-buffered saline (PBS; 5 mM phosphate buffer, 154 mM NaCl, pH/pD 7.4) of varying $D_2O$ content (0–100%) overnight and loaded into thin-walled quartz capillary tubes (Charles Supper Company; Natick, MA) containing PBS with the same $D_2O$ fraction. The capillaries were sealed with wax and enamel and stored at room temperature

until analyzed by neutron diffraction (Suppl. Table S1). During the diffraction experiments, some samples were examined under more than one set of conditions by removing the tissue from the capillary tube, equilibrating it overnight against PBS containing a different volume fraction of $D_2O$ in water, and then resealing it inside a fresh capillary tube.

*Analysis by neutron diffraction*

*Neutron Diffraction:* Neutron diffraction experiments were carried out on the D16 instrument at the Institut Laue-Langevin (Grenoble, France). The monochromatic neutron beam ($\lambda$ = 4.55 Å, $\Delta\lambda/\lambda$ = 0.01) was vertically focused by nine Highly Oriented Pyrolytic Graphite (HOPG) crystals (average mosaic 0.4 deg.), and to optimize sample illumination and angular resolution two pairs of collimating slits were used. The resulting beam size at the sample was 1 mm (horizontal) x 10 mm (vertical). The monochromator-to-sample distance was 2980 mm and the sample-to-detector distance was 955 mm. Diffraction patterns were collected using the Millimeter Resolution Large Area Neutron Detector (MiLAND), a flat, high pressure $^3$He neutron detector with an area of 320 mm x 320 mm and a "pixel" resolution of 1 mm x 1 mm. For all experiments, the detector angle relative to the incident beam (Gamma axis) was set to 5.2 deg, so that it could collect diffraction patterns in a 2θ-range of -4 to 14.6 deg. corresponding to a range in $q$ from -0.096 $Å^{-1}$ to 0.35 $Å^{-1}$, which included the attenuated direct beam, each of the first two order reflections on both sides of the direct beam, and on one side individual 3$^{rd}$ to 5$^{th}$ orders and up to the 7$^{th}$ order reflection.

All neutron diffraction experiments were performed at ambient temperature and pressure, consistent with our previous diffraction experiments at the ILL (Denninger et al., 2014, Denninger et al., 2015). Samples were loaded into thin-walled (20 μm), quartz capillary tubes that were 0.7 mm-diameter for sciatic nerves, and 1.5 mm-diameter for spinal cords (Charles Supper Company, Natick, MA). The tubes were filled with PBS containing a known fraction of $D_2O$ and sealed with wax and enamel. The entubed tissues were mounted and aligned in a dedicated sample changer; and exposure times for data collection ranged from 1–3 h depending on water contrast and whether the sample was sciatic nerve or spinal cord. In the current paper, we report on our structural analysis of the sciatic nerves.

*Neutron Data Reduction:* Data refinement was performed using the ILL in-house software LAMP (Richard et al., 1996; http://www.ill.eu/instruments-support/computing-for-science/data-analysis). First, two-dimensional diffraction patterns were normalized to the incident neutron beam flux to account for variations in beam intensity and exposure time between samples. Second, they were normalized to a detector calibration file containing the flat incoherent signal from water, which is used to correct the

observed intensities for pixel efficiency and solid angle. Third, they were corrected for the attenuation of the direct beam by the sample (transmission). Finally, background patterns from empty quartz capillaries and instrument background were collected and subtracted from each sample pattern and normalization to the sample thickness was applied. The corrected two-dimensional patterns were then integrated vertically (along the neuron axis) to produce one-dimensional diffraction patterns (Figure 1).

*Neutron data analysis:* Peak positions, peak widths, and integrated intensities above background were estimated using MagicPlot software (Magicplot Systems, LLC). Then, for all samples the myelin period (*d*) was calculated according to:

$$d = 2\pi/q \qquad (1)$$

where *q* is the scattering vector given by:

$$q = 4\pi sin\theta/\lambda \qquad (2)$$

where $\lambda$ is the neutron wavelength, and $\theta$ is one-half the scattering angle. Here, for a precise determination of the myelin spacing, the $q_h$ peak positions of each sample were plotted against order *h*. Combining Eq. (2) with Bragg's law ($h\lambda = 2dsin\theta$) yielded the linear dependency between $q_h$ and *h*; and a linear fit to the $q_h$ *vs*. *h* data provided the slope and, therefore, the *d*-spacing (Suppl. Fig. S2).

Structure amplitudes (F(*h*)) were calculated from the peak intensities (I(*h*)) using the equation

$$|F(h)| = (hI(h))^{½}. \qquad (3)$$

Linear regression analysis was performed between F(*h*) and %$D_2O$. Phases were assigned to structure factors according to Kirschner *et al.* (1975), Kirschner and Hollingshead (1980), and Denninger *et al.* (2014). Average neutron scattering density profiles were calculated using F(*h*) according to

$$\rho(r) = \frac{2}{d}\Sigma F(h)cos(\frac{2\pi hr}{d})). \qquad (4)$$

Owing to changes in the widths of the water-accessible spaces in fixed myelin (Kirschner, Hollingshead, 1980), determining the phases for the structure amplitudes measured for the fixed myelin analyzed here involved revising the strategy used previously for native (unfixed) myelin (Kirschner, 1974; Kirschner et al., 1975). Briefly, for tissues from H-dam and H-pups that were not exposed to deuterium-spiked water, a step-function approximation of the contrast between the hydrocarbon of the membrane bilayer and the non-hydrocarbon layers was used to represent the scattering density profile of the myelin: for myelin in 100% $D_2O$-PBS, the major contrast is between the hydrocarbon and the water layers at the cytoplasmic and extracellular appositions of the membrane multilayers; and for myelin in 0% $D_2O$-PBS, the major contrast is between the hydrocarbon and the lipid polar groups. For the step-function approximation, we measured the widths of these layers from the x-ray scattering density profile of

glutaraldehyde-fixed myelin of mouse sciatic nerve, in which the membrane bilayer dimensions have remained constant, whereas the widths of the cytoplasmic and extracellular separations have decreased and increased, respectively (Kirschner & Hollingshead, 1980). We revised the step-function model for unfixed myelin (Kirschner, 1974; Kirschner et al., 1975) by incorporating these altered dimensions of the aqueous spaces (Suppl. Fig. S3). The modified step-function model was used to calculate membrane pair transforms for the fixed myelin in 100%-$D_2O$-PBS and in 0% $D_2O$-PBS (Fig. 2, continuous curves). The agreement between the observed scattering amplitudes and those predicted by the  transform sampled at h/186.1 Å (for *h*=1–5) established the phases for the neutron diffraction data (Fig. 2, data points; Table I).

*Sample preparation for mass spectrometry*

*Homogenization and lipid extraction:* Trigeminal nerves were homogenized in ice-cold 0.1% ammonium acetate in a tissue grinder. Methanol (375 µl) was added to 50 µl homogenate, which was placed into a glass tube with a screw cap, and the tube was vortexed. Further, 1.25 ml MTBE was added and the mixture was incubated for 1 h at RT under agitation. Phase separation was induced by adding 312.5 µl milliQ water. Upon 10 min incubation at RT, the sample was centrifuged at 1000 g for 10 min. The upper organic phase was collected and dried in a vacuum centrifuge at RT. Dried samples were stored at -20°C until further use.

*Thin-layer chromatography:* High-performance thin-layer chromatography (HPTLC) was used to analyze polar and neutral lipids as described previously (Ganser et al., 1988). Lipids were spotted on Partisil LHPK Silica gel 60A plates (Whatman): 300 µg nerve dry weight for polar lipids, and 300 µg nerve dry weight for neutral lipids. A lipid standard mix (polar lipids: non-hydroxy cerebroside, hydroxy cerebroside, non-hydroxy sulfatide, hydroxy sulfatide, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin; neutral lipids: cholesterol esters, triglycerides, diglycerides, cholesterol, free fatty acids, monoglyceride) was spotted on plates at 2.4 µg. Polar lipids were separated with methyl acetate:1-propanol:chloroform:methanol:0.25% KCl:acetic acid (25:25:25:10:9:0.3 by volume). Neutral lipid were first separated with chloroform:methanol:acetic acid (93.1:1.9:0.1 by volume) to a height of 8 cm and then separated to the top with hexane:ethyl ether:acetic acid (89.3:5.7:0.1 by volume). Polar and neutral lipids were visualized with 10% $CuSO_4$, 8% $H_3PO_4$ and heating at 160°C for 13 min. The distribution and relative intensity of the charred lipids (*S*uppl. Fig. S10*)* closely resembled our previous data from mouse nerve lipids (Ganser et al., 1988).

*Lipid analysis by mass spectrometry*

Lipid extracts were resuspended in 1 ml methanol/chloroform (1:1, v/v) and an aliquot of 150 µl was re-dried in a vacuum centrifuge. Finally, the dried lipid sample was reconstituted with 200 µl mobile phase A (acetonitrile: water, 60:40, v/v) and mobile phase B (isopropanol/ acetonitrile, 90:10, v/v) using a ratio of 60:40. 100 µl was transferred into an MS vial. Samples were separated using a CORTECS™ $C_{18}$ column (Waters, 90 Å, 2.7 µm, 2.1 mm X 100 mm).

Lipid extracts were analyzed with an Agilent 6220 mass spectrometer coupled with an Agilent 1200 series HPLC system in both positive and negative ion modes. Instrument calibration was accomplished using a series of Hexakis compounds (Agilent Technologies, Santa Clara, CA: ESI-L Low Concentration Tuning Mix, Part No. G1969-85000, Lot No. LC21022V); and the reference solution (Agilent: API-TOF Reference Mass Solution Kit, Part No. G1969-85001, Lot No. LB48019) consisted of purine ($C_5H_4N_4$; CAS#120-73-0 and hexakis (Hexakis(1h,1h,3h-tetrafluoropropoxy)phosphazine ($C_{18}H_{18}F_{24}N_3O_6P_3$; CAS#58943-98-9). 10 µl of each experimental sample was injected on the column at a flow rate of 350 µl/min, 65°C column temperature, and with the following gradient: 0 min 40% mobile phase B (see above), 1 min 40% B, 11 min 100% B, 15 min 100% B, 16 min 40% B, with 4 min equilibration. Mass spectrometry parameters were as follows: *m/z* range 100-3000, fragmentor 350 V, skimmer 65 V, gas temp 325°C, drying gas 8 l/min, nebulizer 45 psig, capillary 4000 V. As an example, Supplement Fig. S11 shows the chromatogram for a lipid extract (upper panel) with the mass spectra determined for the same retention time range for the control (H-dam) and treated (D-dam) (middle and lower panels, respectively). Identification of lipid monoisotopes in the MS spectra of samples from the H-dam and H-pups was initially based on *m/z* matching using MS-LAMP (Sabareesh, Singh, 2013); and the identification of *m/z* was vetted by comparison with the updated, curated data in two databases (The LIPID MAPS® Lipidomics Gateway, https://www.lipidmaps.org/, Fahy et al., 2007, 2009; and SwissLipids, https://www.swisslipids.org/#/advanced, Aimo et al., 2015). We further vetted the matching of our experimental *m/z* values to those of lipid species reported specifically for mouse neural tissues (cervical dorsal root ganglia: Cheng et al., 2007; CNS myelin: Gopalakrishnan et al., 2013; brain: Angel et al., 2012; brain tissues: Mohammadi et al., 2016; brain tissues: Maganti et al., 2019); cerebellum: Ren et al., 2021). We found only a single published study that reported MS analysis of mouse sciatic nerve lipids (P60; Norrmén et al, 2014); however, as the *m/z* values were not tabulated, we were unable to use their data to additionally vet our findings. Rather, we used the extensive tabulated data for rat (adult, male Wistar) sciatic nerve lipids that were analyzed by imaging MS (Fernández et al., 2016). In our current study, the conditions we used for analyzing the lipids were not sufficient to detect cholesterol, a major myelin constituent, which is usually cationized via alkaline hydrolysis and detected, for example, as ammoniated, lithiated, or sodiated adducts (West, Reid, 2021). Spectral accuracy of our original data was determined using MassWorks (Version 6,0,0,0) software from Cerno Bioscience

(LasVegas, NV 89144) (Wang, Gu, 2010; Kuelh et al., 2021). (The MassWorks software also corrected automatically for the presence in the deuterated lipids of the natural isotope abundance that was detected in the non-deuterated samples (H-dam and H-pup spectra in Fig. 6). Subsequently, we used MassWorks to identify the corresponding deuterated isotopologues in the isolated lipids from the D-mice based on the vetted undeuterated monoisotopes for the isolated lipids from the H-mice, and to quantitate the relative percentage of each isotopologue. The average level of deuteration was calculated by summing these relative percentages (i.e., $\%D = \Sigma_{i>0} A_i$). From the isotopologue distribution we also calculated the weighted average number (and standard error) of deuteriums for each molecular species of lipid (Heckert, Filliben, 2003).

The stated uncertainties in the quantitation of the data are standard deviations, unless indicated otherwise.

## Results and Discussion

### *Use of fixed tissue for diffraction*

Owing to the logistical complexity in raising pregnant dams and their offspring on a strict regimen of defined $D_2O$-$H_2O$ drinking water over a period of several months at the ESRF's animal facility that was accessible to us at the ILL (Grenoble), we undertook the long-duration, preparative portion of the experiment at Boston College. As a result, to maintain as well as possible the structural integrity of the tissue during their hand-carried transit to Grenoble from Boston, we fixed the tissue in aldehyde solutions and stored them in PBS (as described above, Materials and Methods: Sample preparation). Previous experiments that used x-ray diffraction to document the structural changes in myelin during processing for electron microscopy show that the initial fixation step results in relatively small changes in myelin periodicity, membrane packing, and electron density distribution compared to the subsequent steps in tissue processing (Kirschner & Hollingshead, 1980; Avila et al., 2005). Whereas the diffracted intensity is nearly as strong as from unfixed nerves, there are, however, changes in the positions of the Bragg orders and their relative intensities that arise from the narrowing of the cytoplasmic apposition and widening of the extracellular space between membranes. In spite of such well-defined changes in membrane packing caused by chemical fixation, numerous x-ray diffraction studies indicate that intrinsic differences between different types of myelin that are identically fixed may still be detected (Avila et al., 2005; Flores et al., 2008; Avila et al., 2010; Kirschner et al., 2010; da Silva et al., 2014; Luoma et al., 2015).

The neutron diffraction patterns for unfixed (data from Denninger et al., 2014) and fixed (H-dam, this study) mouse sciatic nerves in 100% $D_2O$-PBS show a shift in the positions of the Bragg orders, which is accounted for by the increase in myelin periodicity to 186.1 ± 0.8 Å (n=17) from 175.3 ± 2.2 Å (n=5; Denninger et al., 2014). The first three Bragg orders, which have the strongest intensities at 100% $D_2O$ in both unfixed (see Fig. 1c in Denninger et al., 2014) and fixed myelin (this manuscript, Fig. 1A), dominate the patterns. The main difference is the stronger 1$^{st}$ order intensity in fixed compared to unfixed myelin. Among the higher orders, the main difference is the increase in the 5$^{th}$ and decrease in the 6$^{th}$ order in the fixed compared to the unfixed. These differences in periodicities and intensities between unfixed and fixed myelin are mostly accounted for by the change in packing of the membranes in the myelin array—namely, the widening of the extracellular space and narrowing of the cytoplasmic space between membrane pairs (Suppl. Fig. S5). By contrast, the myelin that had been deuterated via metabolic incorporation from $D_2O$ (and then fixed) showed indistinguishable periodicities (Suppl. Fig. S2) but different intensities compared to the (fixed) non-deuterated tissue (Fig. 1). This suggests that there was an appreciable and specific localization of deuterium that had been incorporated stably into the membrane structure.

*Systematic changes in scattering density of membrane profiles consistent with deuteration of lipids*

To reveal the localization of deuterium in the myelin membrane arrays, we calculated the scattering length density profiles from the phases assigned to the structure amplitudes (see *Materials and Methods*: *Neutron data analysis*). This determination involved use of the step-function defined by the water-accessible spaces of myelin (Suppl. Fig. S3), calculation of membrane transforms from the models to predict structure amplitudes (Fig. 2), and linear regression between the structure amplitudes and %$D_2O$ in the water spaces (Fig. 3) to confirm phase assignments.

The profiles showed that the H-pups and the H-dam corresponded closely in scattering density when the nerves were equilibrated in 0% $D_2O$-PBS or in 100% $D_2O$-PBS (Fig. 4A): i.e., the centers of the membrane bilayers at ~40 Å, the neighboring peaks at ~20 Å and 60 Å, and the inter-bilayer, water-accessible spaces centered at 0 Å and ~90 Å superimposed in both position (x-axis) and level (y-axis). By comparison, after equilibration of nerves in 0% $D_2O$-PBS the scattering density profiles for the D-pups and D-dam superimposed at the peak and trough positions (x-axis), but the density levels (y-axis) for D-pup were significantly higher than those of D-dam except in the water-accessible spaces centered at 0 Å and ~90 Å between membrane bilayers (Fig 4B). Thus, myelin from young mice that

had been exposed to deuterium via $D_2O$ during gestation showed a more pronounced increase in scattering density in the membrane bilayer region compared to their mother, who had also been exposed to the same $D_2O$-spiked drinking water. The greater incorporation of deuterium in the pups is consistent with the greater extent of myelination during gestation and development than during the relative stability of myelin in the mature dam.

Comparison of the scattering density profiles for myelinated nerves from the undeuterated vs. deuterated mice (H-mice vs. D-mice) revealed more clearly the localization of deuterium (Fig. 5). Overall, the two profiles for H-dam and D-dam in 0% $D_2O$-PBS were very similar but not identical (Fig. 5A), as there was a slight but significant increase in scattering density for D-dam in the central hydrocarbon layer (Fig. 5B; see also Suppl. Fig. S9). This suggests that a smaller amount of deuterium had been incorporated into the dam's tissue, most likely owing to the steady-state turnover of membrane constituents during the metabolism that maintains myelin's integrity after maturation. By contrast with the small difference in scattering density profiles between the dams, the difference between the undeuterated and deuterated pups was a substantial elevation of scattering density centered in the membrane bilayer (Fig. 5C,D and Suppl. Fig. S7). This singular feature was evident and uniform across the entire range of contrast variation provided by different % $D_2O$-PBS solutions (Suppl. Figs. S6, S7). Given that lipids are the major hydrogen-rich, non-aqueous constituent of myelin, constituting 70-80% of its dry weight (Schmitt et al., 2014), it is not altogether surprising that metabolically-incorporated deuterium would be most apparent in the H-rich hydrocarbon region of the membrane bilayer. Lipid deuteration would arise from incorporation of deuterium during *de novo* lipogenesis of deuterium from the $D_2O$-spiked drinking water quaffed by the pregnant dam and subsequently by the weaned pups. Studies on the incorporation of deuterium into fats (FA and TG) show that such labeling occurs directly from water, and also via enzyme-catalyzed H-D exchanges during metabolic conversions (Turner et al., 2003; Zhang et al., 2017). We expect that the myelin proteins are also deuterated; however, not only do they constitute only 20–30% of the myelin, but also they are distributed across the entire breadth of the membrane, which includes the intermembrane, polar spaces at the cytoplasmic and extracellular interfaces, and also the nonpolar transmembrane region. It has been estimated that hydrogens are ~50% of all atoms in proteins, whereas they are >60% of all atoms in lipids (and closer to 70% in the hydrocarbon tails). Given the lesser amount of proteins compared to lipids in myelin, and their broader distribution across the membrane, our ability to detect—by neutron diffraction and with the current experimental design—deuterated protein in the presence of deuterated lipid would be challenging.

In addition to the prominent density increase in the hydrocarbon region, the difference maps across the range of contrast variation (Suppl. Fig. S7) show a more subtle difference in deuterium distribution. As the amount of diffusible deuterium increased via H-D exchange from the equilibration solutions that ranged from 0% $D_2O$ to 100% $D_2O$, there was a systematic shift of the high density, metabolically-incorporated deuterium-label peak by 7–8 Å along the x-axis toward the lipid polar group layer in the extracellular half of the bilayer. At the current resolution of the data, this change is within the uncertainty of the error bars; however, it suggests that there are exchangeable hydrogens at this depth in the membrane bilayer, at least for aldehyde-fixed myelin. We conclude from the structural analysis that the metabolically-incorporated deuterium is predominantly concentrated in the central trough of the membrane bilayer, i.e., where there is the greatest proportion of hydrogen atoms that could be substituted for in part by deuterium during de novo lipogenesis or during normal lipid turnover. To gain a more thorough understanding of the nature of the deuteration, we undertook analysis by mass spectrometry of the lipids isolated from the trigeminal nerves that had been frozen in parallel to the sciatic nerves used for structural analysis.

*Metabolic deuteration of mouse tissue lipids*

The earliest research describing the distribution of mass isotopologues among deuterated myelin lipids isolated from $D_2O$-administered mice was reported by Ando et al. (2003). Their protocol was a "pulse-chase" in which 30% $D_2O$ in drinking water was available *ad libitum* for 4–6 weeks to four different groups of mice, ranging in age from five weeks to ~2 years. The mice were sacrificed periodically for up to ~200 days to follow the incorporation and disappearance of deuterium from myelin lipids purified from the CNS (cerebellum). An earlier study (Tanaka & Ando, 1981) focused on the replacement rates of liver lipids (TAG, PC, and PE) in ~6 week old[1] mice in which deuterium from D-spiked drinking water was integrated into fatty acids during a 15-day timespan. Both of these studies demonstrate the feasibility of incorporating deuterium—a stable non-radioactive isotope—to study lipid turnover in mice; however, all the mice including the youngest ones were beyond the age of their most rapid and robust myelination, which is up to ~4 weeks in the PNS and ~5 weeks in the CNS (Agrawal et al., 2009). To distinguish lipogenesis during myelination versus that from myelin maintenance, we initiated $D_2O$ administration via drinking water to pregnant dams, and analyzed the lipids from both the dam and her pups when the latter were mature (P55). In the current paper, we report our MS analysis

[1] Age was deduced from the stated, initial weight of the mice of the particular strain, C3H/He (https://www.criver.com/products-services/find-model/c3h-mouse?region=3611; https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000659)

of trigeminal nerve lipids to illuminate chemically our structural findings on sciatic nerve (another myelinated PNS tissue) from the same mice.

*MS confirmed deuteration of lipids, and variable deuterium levels and label distribution in D-dam*

Negative-ion MS of the lipids isolated from whole trigeminal nerves from the controls—H-dams and their offspring, H-pups—showed the expected natural abundance isotopes $^{1}H$, $^{12}C$, and $^{14}N$ for the monoisotopes (see upper two spectra in each panel, Figs. 6; Suppl. Fig S11, middle). As a control, we also used the MassWorks analytical tool to determine the level of naturally-occuring deuterium ($^{2}H$, D) incorporation in the controls: the value was 0.19% ± 2.4% (n=230), which is about ten times the abundance of $^{2}H$ in water (Kirshenbaum, 1951). Given the large standard deviation, this apparent low level of deuterium found in the controls is statistically indistinguishable from the expected zero value, indicating rather the MS measurement error or noise. By contrast with the MS spectra from the H-mice, all of the spectra from the D-pups and most spectra from the D-dam showed a decrease in the relative intensity of the monoisotopic peak that was paralleled by an increased number of detectable mass isotopologues (Figs. 6, lower two spectra in each panel; Suppl. Fig. S11B). The increase in abundance of the evenly-spaced isotopologues at *m/z* values (A+1, A+2, ...A+n) following the monoisotope peak ($A_0$) indicated the addition of $N$ = 1…..$n$ deuterium atoms to the lipid, or the addition of $M_1$, $M_2$, ..., $M_n$ isotopologues to the original isotopologue $M_0$. The MassWorks analysis clearly resolved the undeuterated isotopologue $M_0$ from the deuterated isotopologues ($M_1$, $M_2$, …, $M_n$) and indicated how much more deuterated the pups' lipids were by contrast with the variable level of deuteration of the dam's lipids: e.g., for the identified lipids, the average level of deuteration in the D-pups' lipids was 97.6% ± 2.0% (n=54; range 89.6–99.6%), whereas their mother's (D-dam's) values ranged broadly from 11.4–97.3% (avg. 60.6% ± 26.4%, n=27) (Fig. 7). Moreover, the number of additional isotopologues was more variable and generally fewer in D-dam. The consistent, overwhelming deuteration of lipids from the D-pups must be a consequence of the prolonged period from early fetal life to two months of age, when deuterium was incorporated metabolically into the rapidly myelinating tissues (Agrawal et al., 2009) and when there would likely be very slow turnover of lipids in the growing, multilamellar myelin sheath. During this same timeframe for treatment, the mother would have maintained a normal level of lipid turnover, which would depend not only on the particular lipid species but also on the molecular subspecies. This interpretation assumes that the deuterium effect on biosynthesis and assembly, and on metabolism and turnover is negligible, although the C-D bond does have a higher dissociation energy (341.4 kJ/mol vs. 338.4 for  kJ/mol for C-H; Luo, 2007) and is

reported to be less susceptible to enzymatic cleavage (Kushner et al, 1999). Our finding for mouse PNS myelin is consistent with results from analysis of mouse and rat CNS myelin, which have been shown to undergo appreciable changes not only across phospholipid and glycolipid species but also among their molecular subspecies during development and maturation (Leray et al., 1994; Davis et al., 2020).

Based on the MS analysis of neural tissues from mice (and rats) and from curated databases, we identified 26 distinct lipids distributed over three different lipid classes (Fig. 6) in our samples. The classes consisted of glycerophospholipids (with the species PE, PC, PS, and PI), sphingolipids (species HexCer, SHexCer, SM), and acylglycerols (species FA, DG, and TAG). Except for PI and the acylglycerols, each lipid species had more than one molecular subspecies. To compare lipids, we arranged the MS spectra by molecular subspecies according to the deuterium distribution patterns exhibited by D-dam, such that lipids with similar patterns were grouped together into three categories based on high (Group 1), intermediate (Group 2), and low levels of deuteration (Group 3), indicative of high, intermediate, and low turnover of lipids. The most heavily deuterated lipids (>90%) were PC (three of seven subspecies), PI, and DG, and closely resembled the corresponding spectra of the pups. The intermediate levels of deuteration (~60–90%) also resembled that in the pups but at lower levels, and consisted of PE (four of six subspecies), PC (three of seven subspecies), and PS (one species). The least deuterated (~10 to <60%), and hence most stable lipids, were SHexCer (three subspecies), TG, SM (two), PE (two of six), FA, HexCer (two), PC (one of seven), and PS (one of two).

Our results are consistent with previous findings that used precursors labeled with a variety of tracers ($^{13}C$, $^{14}C$, $^{2}H$, $^{3}H$, $^{32}P$) and demonstrated, at least in the CNS, a differential distribution of label and widely differing turnover rates among the lipid classes of myelin. For example, there is general agreement that the ranking of mouse myelin lipids from most rapid to slowest turnover half-life is: (PI, PC) ≈ PE < (HexCer, SHexCer) < SM (Morell, Ousley, 1994; Ando et al., 2003; Poitelon, Belin, 2020; and references therein).

What is distinctive about our results compared to the earlier studies is the clear variation in deuteration levels among the molecular subspecies of the lipids we identified: e.g., the seven subspecies of PC varied from ~50% to >90%, and the six molecular subspecies of PE varied from ~40% to ~80%, indicating a wide range of metabolic turnover. In addition, all three subspecies of SHexCer had very low levels of deuteration (varying form ~10% to <30%), reflecting long half-lives—stability—in the myelin membrane, whereas HexCer (the precursor for sulfatide) showed ~50% deuteration and, therefore, greater turnover. The similarly-high deuteration levels (at >90%) of PI and DG is consistent with their

role in the inositol--phospholipid signaling pathway, where hydrolysis of the membrane-bound, doubly-phosphorylated PI (or PIP2) forms two second messengers, one of which (DG) remains in the membrane. The deuterated fatty acyl chains of PI, therefore, most likely account for the deuterated fatty acyl chains of DG. The low level of deuteration for TG (~20%) is consistent with its role as a stable, storage reservoir for fatty acids. The high deuteration level for several of the PC subspecies that we identified may relate to the biosynthetic pathway involving condensation between highly-deuterated DG and cytidine 5'-diphosphocholine (CDP-choline or citicoline) (Fagone and Jackowski, 2013). PC also plays a role in membrane-mediated cell signaling and phosphatidylcholine-transfer protein activation of other enzymes (Kand et al., 2010). Taken together, our findings underscore not only the dynamic metabolic diversity of the lipids but also are consistent with widely varying functions among their subspecies.

As described above, deuterium enrichment was indicated by the progressive increase in the abundance of high mass isotopologues. These labeled molecules always formed symmetric, bell-shaped envelopes for the lipids of D-pups, but usually formed more complex envelopes for lipids of the D-dam. The symmetric shape of the envelope for D-pups indicated a maximum level of deuteration for the D-pups' lipids, which depends on the relative amount of deuterium and hydrogen in the $D_2O$-spiked drinking water. Exceptions were SHexCer (m/z 862.61) and PC (m/z 844.68) which showed slightly higher proportions of lower-mass isotopologues (e.g., $M_1$ and $M_2$), that might be explained by different "schedules" of lipogenesis for these two lipid subspecies during gestation: e.g., these particular molecular subspecies of sulfatide and phosphatidylcholine might be the earliest synthesized by the myelin-forming cells of the gestating pups and thus crucial to subsequent myelin formation. There is abundant evidence in the CNS that sulfatide has crucial roles in oligodendrocyte maturation, and subsequently in myelin formation and stability (reviewed by Grassi et al., 2016); and sulfatide subspecies have been shown to have different developmental timetables during oligodendrocyte maturation and myelinogenesis as demonstrated using imaging mass spectrometry (Hirahara et al., 2017). Other distinct distributions were PE (m/z 700.53) and PC (m/z 758.57) which showed enhanced values for higher-mass isotopologues $M_i$ (e.g., $i > 10$), suggesting a different population of these subspecies; however, these variations may also be accounted for by slight differences in the retention times (see Suppl. Fig.11).

The average number of deuterium labels in a lipid, calculated as a weighted average, depended on the lipid species (Fig. 8). By contrast with results from D-pups, for D-dam the shapes of the isotopologue distribution curves ranged from being symmetrical, bell-shaped—Group 1 of D-dam (DG, PI, and PCs

having m/z values of 806.57, 885.55, and 758.57 [the lattermost showing two, overlapping bell-shaped patterns])—to showing a complex combination of high-percentage monoisotope with nascent bell-shaped isotopologue distributions (many in Group 2), to being predominantly undeuterated with relatively small levels of label (Group 3). Similar isotopologue distributions were also reported for cerebellar myelin lipids (Ando et al. 2003) and for liver fatty acids (Tanaka, Ando, 1981; Ando, Tanaka, 2005) from mice given $D_2O$-spiked drinking water. These researchers accounted for their findings by positing the coexistence of several fractions of molecules that included newly-synthesized ones having relatively few deuteriums, to molecules that were either undeuterated or synthesized from recycled deuterium-containing constituents. Reactions that are likely to be involved in incorporating deuterium from $D_2O$ into the lipids include glyceroneogenesis or glycolysis, where deuterium can be transferred into the C-H bonds of tryglceride's glycerol backbone (Turner et al., 2003), and the fatty acid synthesis reaction, where deuterium can be incorporated enzymatically into the lengthening fatty acyl chains (Zhang et al., 2017; Belew et al., 2019).

**Conclusions**

In our study, the pups were sacrificed at P55, which is immediately after the most rapid rate of myelination; whereas the dams were sacrificed at P131, near the end of the slowed rate of myelination which is about 4% the initial phases of robust accretion of lipid-rich membranes (Agrawal et al., 2009). During this phase of myelin maintenance, when there is less *de novo* synthesis of lipids, the relative metabolic stability in the mature myelin of the dam accounts for the lower level of deuteration of many of her lipids, but at the same time some of her lipids show a high rate of turnover—e.g., many of the PCs, PI and DG are highly deuterated owing to their greater functional demands.

Our study's accomplishments were twofold: one, we demonstrated that neutron diffraction could successfully localize in intact tissue deuterium that had been metabolically incorporated *in vivo*; and two, we demonstrated that metabolic incorporation of deuterium as a stable isotope during gestation vs. after maturation can provide insight about the fates of specific lipid species and subspecies in myelin formation, maintenance, and turnover. Based on these findings, we anticipate exploitation of this approach to developing a comprehensive understanding of how lipids are differentially regulated throughout life—gestation, the three phases of myelin accumulation, myelin "stability" during its maintenance, and myelin aging—in normal and pathological tissue.

**Future Perspectives**

Our approach has implications for further research on myelin and more broadly in other areas. In particular, relevant to myelin, we can ask the following questions: What functions account for the differential deuteration of PC subspecies in the dam? How do the turnover rates for the different lipids vary with age once the myelin is "fully" mature? When the balance in myelin formation and maintenance are upset owing to mutations in lipid biogenic enzymes or in myelin structural proteins, how are the lipid species turnovers affected? If lipids in the extracellular leaflet are less subject to turnover and more stable metabolically, can we argue that the least-deuterated lipids that we identified in D-dam are in the extracellular half of the bilayer—e.g., SHexCer, TG, and SM? and that the most highly-deuterated lipids in D-dam are in the more metabolically-accessible cytoplasmic half of the bilayer—e.g., PC (three subspecies), PI, and DG? Thus, the convergence of lipidomics and metabolomics can be anticipated to provide extraordinary insight into myelinomics. The analytic and quantitative power of this approach has been demonstrated using HeLa cells (Goh et al., 2018; Kim et al., 2019). Further, in the experimental paradigm we used here for stable isotope incorporation via $D_2O$-spiked drinking water, not only was myelin labeled, but one can surmise a priori that all tissues in the mice were deuterated, and differentially for the pups vs. the dam. Tissues that exhibit supra- and macromolecular order, including muscle fibers, retinal rod outer segments, collagen fibers, bone, etc., or proteins that could be isolated and organized with crystalline or paracrystalline order, e.g., hemoglobin, numerous enzymes, β-amyloid, etc., could be informative assemblies for analysis by neutron diffraction. "Harvesting" these at different times during *in vivo* deuteration or after the $D_2O$ has been deleted from the drinking water, and analyzing by MS the changes in distribution patterns of deuterium labels would illuminate our understanding of the dynamics of these tissues and molecular assemblies. In short, we anticipate that our correlated structural and biochemical study will promote a broad, comprehensive approach that is informative about structure, function, and metabolism in both health and disease.

**Acknowledgements**

We thank Dr. Giuseppe (Joe) Zaccai for his continued interest and encouragement, The Western Norway Regional Health Authorities (Helse Vest project 911957) for providing Travel Support for AB, The Company of Biologists Ltd. (Histon, Cambridge, CB24 9LF, UK) for a Travel Fellowship awarded to ARD, Boston College for an Ignite Research Award to DAK, the Mass Spectrometry Facility at Boston College's Eugene F. Merkert Chemistry Center, and Dr. Vivian Cristiglio for on-site technical support at the D16 neutron beamline. We thank the ILL for their generosity in allocating beam time on the D16 (corresponding data DOI: 10.5291/ILL-DATA.8-02-734), and in hosting DAK in 2017 as a Visiting Scientist. We are particularly grateful to Dr. Yondong Wang, Cerno Bioscience (Las Vegas, NV), for his encouragement, and generous contribution and tutelage in using MassWorks. We also acknowledge useful discussions with Dr. Michael Kiebish (BERG Health LLC) in the early stages of our MS analysis. DAK dedicates this paper to the memory of Dr. Donald L.D. Caspar (1927-2021), who was his graduate school and postdoctoral research mentor, and who remained keenly interested in DAK"s experimental findings in the research areas of structural biology pertaining to myelin and amyloid.

**Conflicts of interest**

None.

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## Tables

### Table 1: Structure-factor for D-pup, D-dam, H-pup and H-dam versus %$D_2O$.

Values are extrapolated (except for D-dam) from the linear fit of the experimental data and normalized. No standard errors were determined for high Bragg orders (*h*=6) if only two experimental data points were available (see Fig. 3).

| | | Extrapolated and normalized F(*h*) ± standard error | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Bragg order *h* | 0 % $D_2O$ | 20 % $D_2O$ | 30 % $D_2O$ | 40 % $D_2O$ | 60 % $D_2O$ | 80 % $D_2O$ | 100 % $D_2O$ |
| D-pup | 1 | 0.1476 ± 0.0035 | 0.0223 ± 0.0037 | -0.0404 ± 0.0039 | -0.1030 ± 0.0043 | -0.2283 ± 0.0051 | -0.3535 ± 0.0060 | -0.4788 ± 0.0070 |
| | 2 | -0.1500 ± 0.0102 | 0.0800 ± 0.0110 | 0.1950 ± 0.0119 | 0.3100 ± 0.0131 | 0.5400 ± 0.0160 | 0.7700 ± 0.0193 | 1.0000 ± 0.0229 |
| | 3 | 0.0246 ± 0.0083 | 0.1068 ± 0.0086 | 0.1479 ± 0.0089 | 0.1889 ± 0.0093 | 0.2711 ± 0.0104 | 0.3532 ± 0.0117 | 0.4354 ± 0.0132 |
| | 4 | -0.0837 ± 0.0157 | -0.0755 ± 0.0169 | -0.0714 ± 0.0182 | -0.0673 ± 0.0200 | -0.0590 ± 0.0243 | -0.0508 ± 0.0292 | -0.0426 ± 0.0346 |
| | 5 | -0.1306 ± 0.0126 | -0.0875 ± 0.0133 | -0.0659 ± 0.0141 | -0.0444 ± 0.0151 | 0.0012 ± 0.0176 | 0.0419 ± 0.0207 | 0.0850 ± 0.0241 |
| | 6* | | | | | | | 0.0591 ± 0.0038 |
| | 7* | -0.0497 ± 0.0087 | | | | | | -0.0640 ± 0.0055 |
| D-dam* | 1 | 0.0846 ± 0.0032 | | | | | | |
| | 2 | -0.0659 ± 0.0023 | | | | | | |
| | 3 | 0.0765 ± 0.0023 | | | | | | |
| | 4 | -0.0862 ± 0.0033 | | | | | | |
| | 5 | -0.1259 ± 0.0018 | | | | | | |
| | 6 | | | | | | | |
| | 7 | -0.0497 ± 0.0087 | | | | | | |
| H-pup | 1 | 0.0644 ± 0.0099 | -0.0432 ± 0.0106 | -0.0970 ± 0.0114 | -0.1508 ± 0.0124 | -0.2584 ± 0.0149 | -0.3660 ± 0.0179 | -0.4736 ± 0.0210 |
| | 2 | -0.0018 ± 0.0224 | 0.1986 ± 0.0243 | 0.2988 ± 0.0264 | 0.3989 ± 0.0291 | 0.5993 ± 0.0358 | 0.7996 ± 0.0434 | 1.0000 ± 0.0515 |
| | 3 | 0.0893 ± 0.0115 | 0.1543 ± 0.0121 | 0.1867 ± 0.0128 | 0.2192 ± 0.0137 | 0.2841 ± 0.0160 | 0.3490 ± 0.0188 | 0.4140 ± 0.0218 |
| | 4 | -0.0746 ± 0.0091 | -0.0727 ± 0.0098 | -0.0718 ± 0.0107 | -0.0709 ± 0.0117 | -0.0690 ± 0.0144 | -0.0672 ± 0.0174 | -0.0653 ± 0.0207 |
| | 5 | -0.1190 ± 0.0150 | -0.0782 ± 0.0160 | -0.0578 ± 0.0172 | -0.0374 ± 0.0187 | 0.0034 ± 0.0225 | 0.0442 ± 0.0269 | 0.0851 ± 0.0316 |
| | 6 | -0.0457 ± 0.0019 | -0.0216 ± 0.0020 | -0.0096 ± 0.0021 | 0.0025 ± 0.0022 | 0.0266 ± 0.0026 | 0.0507 ± 0.0031 | 0.0749 ± 0.0036 |
| H-dam | 1 | 0.0649 ± 0.0147 | -0.0391 ± 0.0158 | -0.0910 ± 0.0171 | -0.1430 ± 0.0187 | -0.2470 ± 0.0227 | -0.3509 ± 0.0274 | -0.4549 ± 0.0324 |
| | 2 | -0.0252 ± 0.0287 | 0.1798 ± 0.0305 | 0.2824 ± 0.0325 | 0.3849 ± 0.0351 | 0.5899 ± 0.0418 | 0.7950 ± 0.0496 | 1.0000 ± 0.0581 |
| | 3 | 0.0763 ± 0.0165 | 0.1398 ± 0.0175 | 0.1716 ± 0.0187 | 0.2034 ± 0.0202 | 0.2669 ± 0.0239 | 0.3304 ± 0.0284 | 0.3940 ± 0.0333 |
| | 4 | -0.0671 ± 0.0004 | -0.0700 ± 0.0005 | -0.0714 ± 0.0005 | -0.0728 ± 0.0005 | -0.0757 ± 0.0006 | -0.0786 ± 0.0007 | -0.0815 ± 0.0008 |
| | 5 | -0.1017 ± 0.0009 | -0.0641 ± 0.0010 | -0.0453 ± 0.0011 | -0.0266 ± 0.0012 | 0.0110 ± 0.0014 | 0.0485 ± 0.0017 | 0.0861 ± 0.0020 |
| | 6 | -0.0394 | -0.0163 | -0.0048 | 0.0068 | 0.0299 | 0.0530 | 0.0761 |
| | 7* | -0.0537 ± 0.0077 | | | | | | -0.1442 ± 0.0037 |

* Experimental data

**Table 2: Identified lipids in mouse trigeminal nerves using LC-MS.**

Ions in the spectra are identified based on m/z matching using LIPID MAPS (The LIPID MAPS® Lipidomics Gateway, https://www.lipidmaps.org/, Fahy et al., 2007, 2009). Tolerance was set to ± 0.02 m/z to reduce the number of matches, which were further vetted by literature. Identified lipids shown in bold were further analyzed.

| | [M-H]- | | | | |
|---|---|---|---|---|---|
| Experimental m/z* | Delta m/z** | Lipid class | Abbrev. | Formula | Reference |
| **700.5286** | **0.0001** | **Glycerophosphoethanolamines** | **PE** | **C39H76NO7P** | **[1] [3] [4] [9]** |
| **726.5449** | **0.0006** | **Glycerophosphoethanolamines** | **PE** | **C41H78NO7P** | **[1] [3] [4] [5] [9]** |
| | 0.0077 | Neutral glycosphingolipids | HexCer | C41H77NO9 | |
| **728.5595** | **0.0005** | **Glycerophosphoethanolamines** | **PE** | **C41H80NO7P** | **[1] [3] [4] [5] [9]** |
| **742.5391** | **0.0001** | **Glycerophosphoethanolamines** | **PE** | **C41H78NO8P** | **[1] [3] [4] [5] [8] [9]** |
| | 0.0084 | Neutral glycosphingolipids | HexCer | C41H77NO10 | |
| **750.5438** | **0.0005** | **Glycerophosphoethanolamines** | **PE** | **C43H78NO7P** | **[1] [3] [4] [5] [7] [9]** |
| 756.5909 | 0.0004 | Glycerophosphoethanolamines | PE | C43H84NO7P | [1] [3] [4] [9] |
| | 0.0086 | Neutral glycosphingolipids | HexCer | C43H83NO9 | |
| 778.5457 | 0.0065 | Glycerophosphoethanolamines | PE | C44H78NO8P | [3] [4] |
| **786.529** | **0.0001** | **Glycerophosphoserines** | **PS** | **C42H78NO10P** | **[3] [4] [5] [7] [8]** |
| **788.5457** | **0.0010** | **Glycerophosphoserines** | **PS** | **C42H80NO10P** | **[1] [3] [4] [5] [6] [7] [8]** |
| 802.5605 | 0.0001 | Glycerophosphoserines | PS | C43H82NO10P | [4] |
| | 0.0151 | Glycerophosphoethanolamines | PE | C47H82NO7P | [3] |
| 804.5761 | 0.0001 | Glycerophosphoserines | PS | C43H84NO10P | [4] |
| | 0.0152 | Glycerophosphoethanolamines | PE | C47H84NO7P | |
| 826.5607 | 0.0003 | Glycerophosphoserines | PS | C45H82NO10P | [4] |
| 828.6569 | 0.0081 | Glycerophosphoethanolamines | PE | C47H92NO8P | [4] |
| 832.6079 | 0.0006 | Glycerophosphoserines | PS | C45H88NO10P | [1] [4] |
| 844.6392 | 0.0045 | Glycerophosphoserines | PS | C47H92NO9P | [3] [4] |
| 854.6729 | 0.0085 | Glycerophosphoethanolamines | PE | C49H94NO8P | |
| 856.6879 | 0.0004 | Neutral glycosphingolipids | HexCer | C49H95NO10 | |
| | 0.0078 | Glycerophosphoethanolamines | PE | C49H96NO8P | |
| **857.6741** | **0.0076** | **Triradylglycerols◆◆** | **TG** | **C56H90O6** | **[4]** |
| **862.6077** | **0.0007** | **Acidic glycosphingolipids** | **SHexCer** | **C46H89NO11S** | **[1] [2] [3] [4] [5] [6] [7] [8]** |
| | 0.0102 | Phosphosphingolipids | SM | C46H90NO11P | |
| | 0.0184 | Neutral glycosphingolipids | HexCer | C46H89NO13 | |
| **885.5504** | **0.0005** | **Glycerophosphoglucose lipids** | **PI** | **C47H83O13P** | **[1] [3] [4] [5] [6] [7] [8]** |
| **888.6234** | **0.0006** | **Acidic glycosphingolipids** | **SHexCer** | **C48H91NO11S** | **[1] [3] [4] [5] [6] [7] [8]** |
| | 0.0184 | Neutral glycosphingolipids | HexCer | C48H91NO13 | |
| **890.6388** | **0.0009** | **Acidic glycosphingolipids** | **SHexCer** | **C48H93NO11S** | **[1] [3] [4] [5] [6] [7] [8]** |
| | 0.0186 | Neutral glycosphingolipids | HexCer | C48H93NO13 | |

| | [M-H]+ | | | | |
|---|---|---|---|---|---|
| Experimental m/z* | Delta m/z** | Lipid class | Abbrev. | Formula | Reference |
| 121.05 | 0.0005 | Fatty Acids and Conjugates | FA or Purine | C4H8O4 | |
| **369.3523** | **0.0160** | **Fatty Acids and Conjugates** | **FA** | **C23H44O3** | |
| 391.2849 | 0.0006 | Bile acids and derivatives | St | C24H38O4 | |
| **627.5346** | **0.0363[a]** | **Diradylglycerols** | **DG** | **C40H66O5** | |
| | **0.0363[a]** | **Fatty Acids and Conjugates** | **FA** | **C40H66O5** | |
| **702.5439** | **0.0007** | **Glycerophosphoethanolamines** | **PE** | **C39H76NO7P** | **[1] [4] [9]** |
| | 0.0007 | Glycerophosphocholines | PC | C39H76NO7P | [4] [9] |
| 705.5011 | 0.0054 | Glycerophosphoglycerols | PG | C38H73O9P | |
| | 0.0157 | Glycerophosphates | PA | C41H69O7P | |
| 706.5429 | 0.0048 | Glycerophosphocholines | PC | C38H76NO8P | [4] [8] |
| | 0.0048 | Glycerophosphoethanolamines | PE | C38H76NO8P | [4] |
| 728.5595 | 0.0006 | Glycerophosphocholines | PC | C41H78NO7P | [9] |
| | 0.0006 | Glycerophosphoethanolamines | PE | C41H78NO7P | [1] [9] |
| | 0.0076 | Neutral glycosphingolipids | HexCer | C41H77NO9 | |
| 730.5752 | 0.0007 | Glycerophosphocholines | PC | C41H80NO7P | [1] [9] |
| | 0.0007 | Glycerophosphoethanolamines | PE | C41H80NO7P | |
| **732.5545** | **0.0007** | **Glycerophosphocholines** | **PC** | **C40H78NO8P** | **[1] [5] [6] [8] [9]** |
| | 0.0007 | Glycerophosphoethanolamines | PE | C40H78NO8P | |
| | 0.0075 | Neutral glycosphingolipids | HexCer | C40H77NO10 | |

| | | | | | |
|---|---|---|---|---|---|
| 744.5561 | 0.0023 | Glycerophosphocholines | PC | C41H78NO8P | [9] |
| | 0.0023 | Glycerophosphoethanolamines | PE | C41H78NO8P | [1] [9] |
| **758.569** | **0.0004** | **Glycerophosphocholines** | **PC** | **C42H80NO8P** | **[1] [2] [5] [6] [8] [9]** |
| | 0.0004 | Glycerophosphoethanolamines | PE | C42H80NO8P | |
| 758.606 | 0.0002 | Glycerophosphoethanolamines | PE | C43H84NO7P | [1] [9] |
| | 0.0002 | Glycerophosphocholines | PC | C43H84NO7P | |
| | 0.0081 | Neutral glycosphingolipids | HexCer | C43H83NO9 | |
| **760.586** | **0.0009** | **Glycerophosphocholines** | **PC** | **C42H82NO8P** | **[1] [2] [5] [6] [8] [9]** |
| | 0.0009 | Glycerophosphoethanolamines | PE | C42H82NO8P | |
| | 0.0073 | Neutral glycosphingolipids | HexCer | C42H81NO10 | |
| 774.5974 | 0.0033 | Glycerophosphocholines | PC | C43H84NO8P | [8] |
| | 0.0033 | Glycerophosphoethanolamines | PE | C43H84NO8P | [1] [8] |
| **782.569** | **0.0004** | **Glycerophosphocholines** | **PC** | **C44H80NO8P** | **[1] [5] [6] [8] [9]** |
| | 0.0004 | Glycerophosphoethanolamines | PE | C44H80NO8P | |
| | 0.0148 | Phosphosphingolipids | SM | C40H80NO11P | |
| 788.5514 | 0.0078 | Glycerophosphoserines | PS | C42H78NO10P | |
| **788.617** | **0.0006** | **Glycerophosphocholines** | **PC** | **C44H86NO8P** | **[1] [2] [5] [6] [8] [9]** |
| | 0.0006 | Glycerophosphoethanolamines | PE | C44H86NO8P | [9] |
| | 0.0076 | Neutral glycosphingolipids | HexCer | C44H85NO10 | |
| 792.6276 | 0.0163 | Glycerophosphoserines | PS | C43H86NO9P | |
| 798.6819 | 0.0002 | Neutral glycosphingolipids | HexCer | C47H91NO8 | [1] [4] |
| **806.57** | **0.0006** | **Glycerophosphocholines** | **PC** | **C46H80NO8P** | **[1] [5] [6] [9]** |
| | 0.0006 | Glycerophosphoethanolamines | PE | C46H80NO8P | |
| | 0.0076 | Neutral glycosphingolipids | HexCer | C42H79NO13 | |
| **810.6821** | **0.0004** | **Neutral glycosphingolipids** | **HexCer** | **C48H91NO8** | **[1] [4]** |
| **812.6975** | **0.0001** | **Neutral glycosphingolipids** | **HexCer** | **C48H93NO8** | **[1] [4]** |
| **813.6852** | **0.0008** | **Phosphosphingolipids** | **SM** | **C47H93N2O6P** | **[1] [4] [6] [8] [9]** |
| | 0.0115 | Triradylglycerols | TG | C52H92O6 | |
| **815.7007** | **0.0007** | **Phosphosphingolipids** | **SM** | **C47H95N2O6P** | **[1] [4] [8] [9]** |
| | 0.0116 | Triradylglycerols | TG | C52H94O6 | |
| **844.6793** | **0.0003** | **Glycerophosphocholines** | **PC** | **C48H94NO8P** | **[1] [4] [6] [8] [9]** |
| | 0.0003 | Glycerophosphoethanolamines | PE | C48H94NO8P | |
| | 0.0079 | Neutral glycosphingolipids | HexCer | C48H93NO10 | |
| 872.7107 | 0.0004 | Glycerophosphocholines | PC | C50H98NO8P | [8] |
| | 0.0078 | Neutral glycosphingolipids | HexCer | C50H97NO10 | |
| 874.6582 | 0.0050 | Glycerophosphoserines | PS | C48H92NO10P | |
| 890.6377 | 0.0009 | Acidic glycosphingolipids | SHexCer | C48H91NO11S | |
| | 0.0186 | Neutral glycosphingolipids | HexCer | C48H91NO13 | |

* Experimental values of m/z identified as particular Lipid or Lipid class using LMSD (The LIPID MAPS® Lipidomics Gateway, https://www.lipidmaps.org/)

** tolerance ±0.02 m/z

¤ included in the list despite the higher delta value, to complement the TLC results (Suppl. Fig. S10)

◆◆ the moniker "triradylglycerol" refers to any one of the substituent groups (acyl, alkyl, or alk-1-enyl) being at each of the three possible positions


[1] Gopalakrishnan *et al.* 2013

[2] Mohammadi *et al.* 2016

[3] Cheng *et al.* 2007

[4] Aimo *et al.* 2015

(5) Maganti *et al.* 2019

[6] Anderson *et al.* 2013

[7] Angel *et al.* 2012

[8] Fernández *et al.* 2016

[9] Ren *et al.* 2021

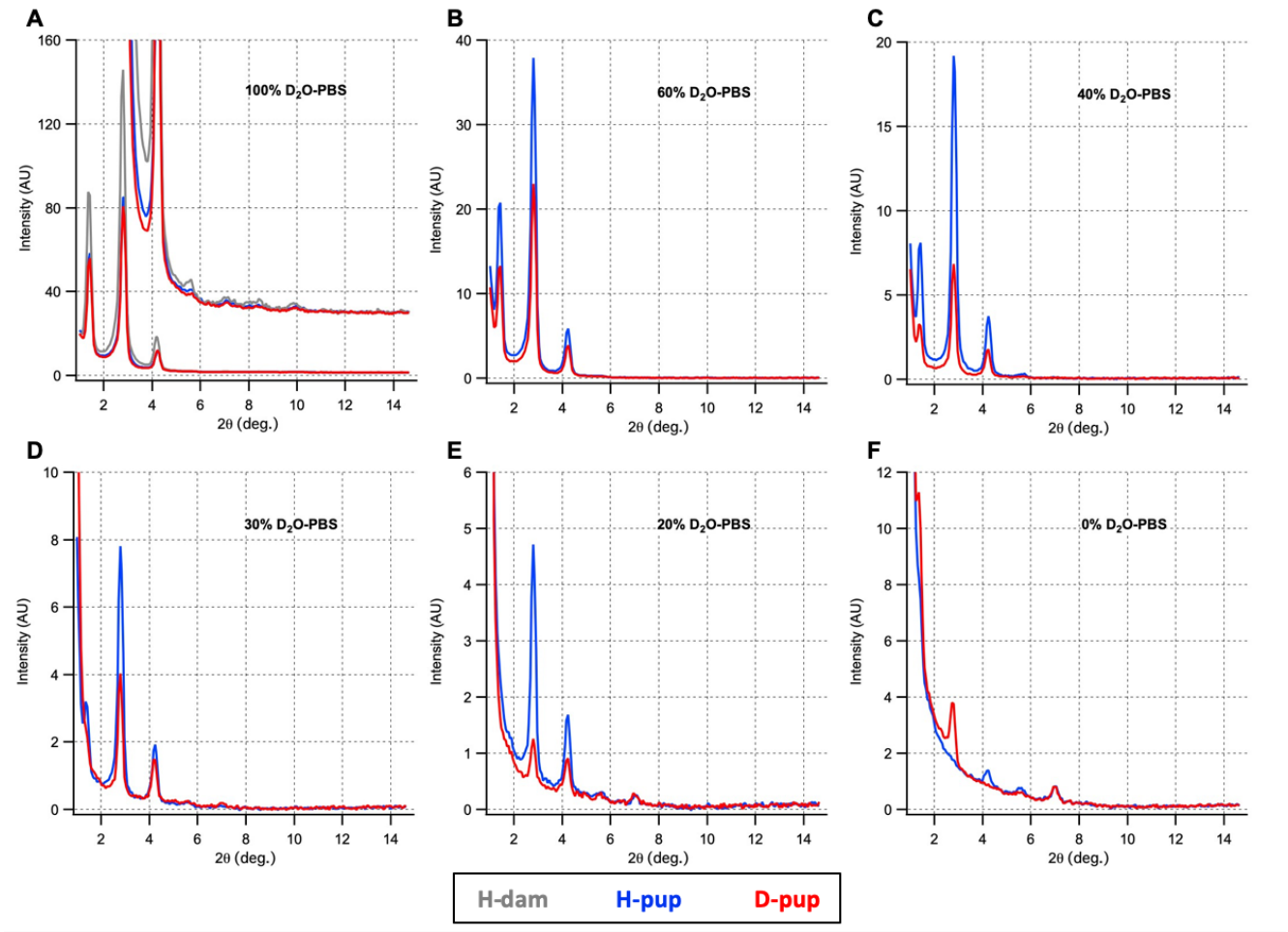



**Figure 1. Neutron diffraction spectra from the (fixed) sciatic nerves of 2 month old pups that had gestated and then been raised on either normal drinking water (blue) or on a mixture of $D_2O$ in water (red) (see Materials and Methods for protocol).**

Nerves were equilibrated in 0%–100% $D_2O$-PBS. The scattering intensity, in arbitrary units (AU), is plotted as a function of scattering angle $2\theta$ in degrees. At 100%–30% $D_2O$-PBS (**A-D**), Bragg orders 1–3 are clearly evident. For each type of pup, the relative intensities change systematically as the %$D_2O$ changes, and these changes differ between the D- vs. H-pups. (**A**) includes the diffraction spectrum from H-dam (grey), the non-deuterated mother of the H-pups, showing the similarity in relative intensities of the Bragg peaks between mother and pup, but nearly twice as strong for the mother. (**B-F**) show that as the amount of diffusible $D_2O$ decreases, the diffracted intensities change more drastically in the deuterated pup (D-pup) compared to the non-deuterated pup (H-pup), owing to localization of the non-diffusible and stable, metabolically-incorporated deuterium in the former. (Diffraction from H-dam in 100%, 40%, and 20% $D_2O$-PBS is shown in Suppl. Fig. S4.)

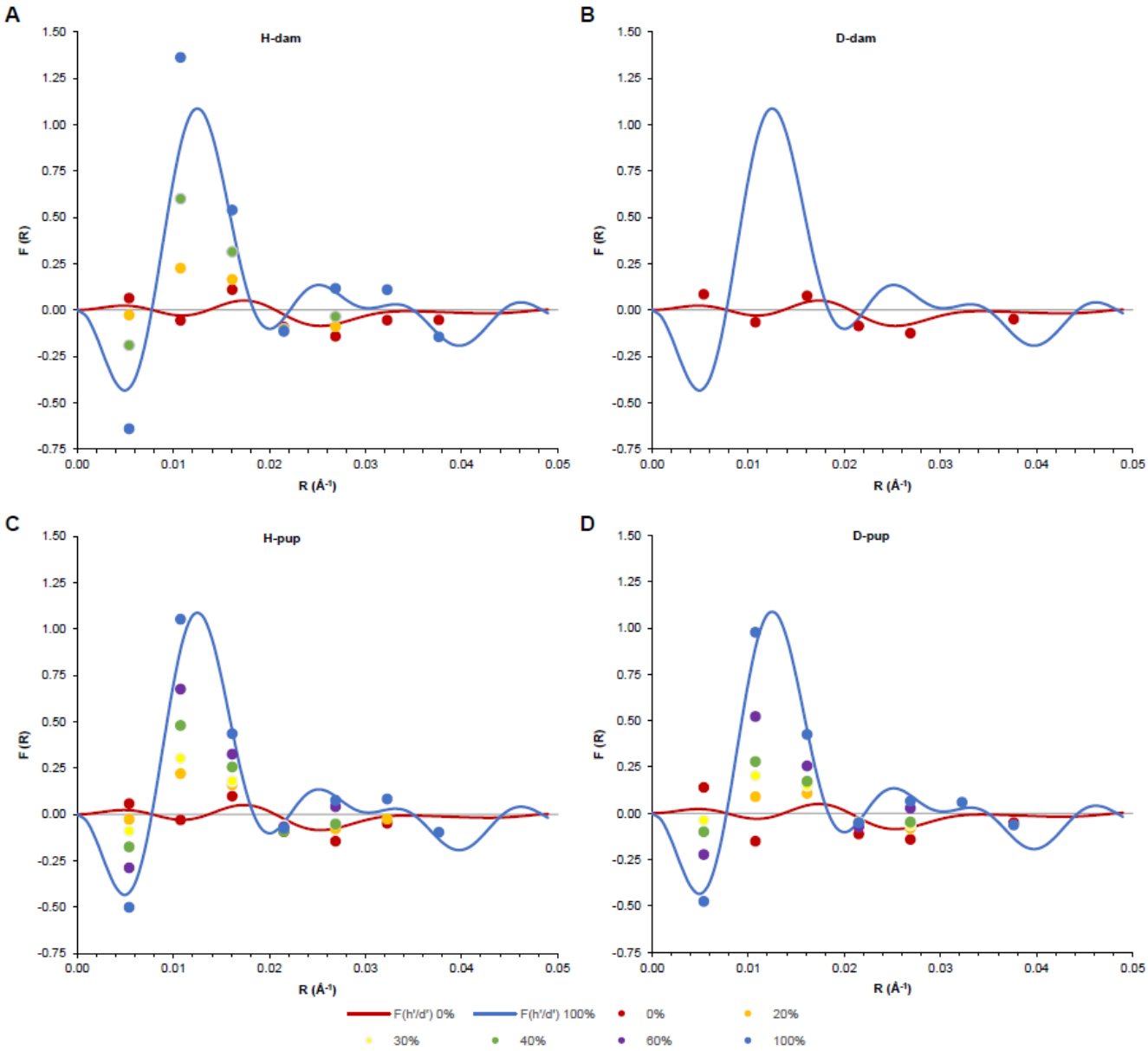



**Figure 2. Determination of phases from step-function.**

The continuous transforms F(R) for H-dam at 100% $D_2O$-PBS and at 0% $D_2O$-PBS were calculated as described (see *Materials & Methods: Neutron Data Analysis*, and Suppl. Fig. S3) and used to predict phases for the structure amplitudes calculated from the Bragg orders. Experimental data points for H-dam (**A**), H-pup (**C**), D-dam (**B**), and D-pup (**D**) at different amounts of $D_2O$ in PBS (0%, 20%, 30%, 40%, 60%, 80%, and 100%) were mapped onto the continuous transform, and showed a systematic change in F(*h*). Linear regression between F(*h*) and %$D_2O$ (Fig. 3) supports the phase assignments based on the step-function models.

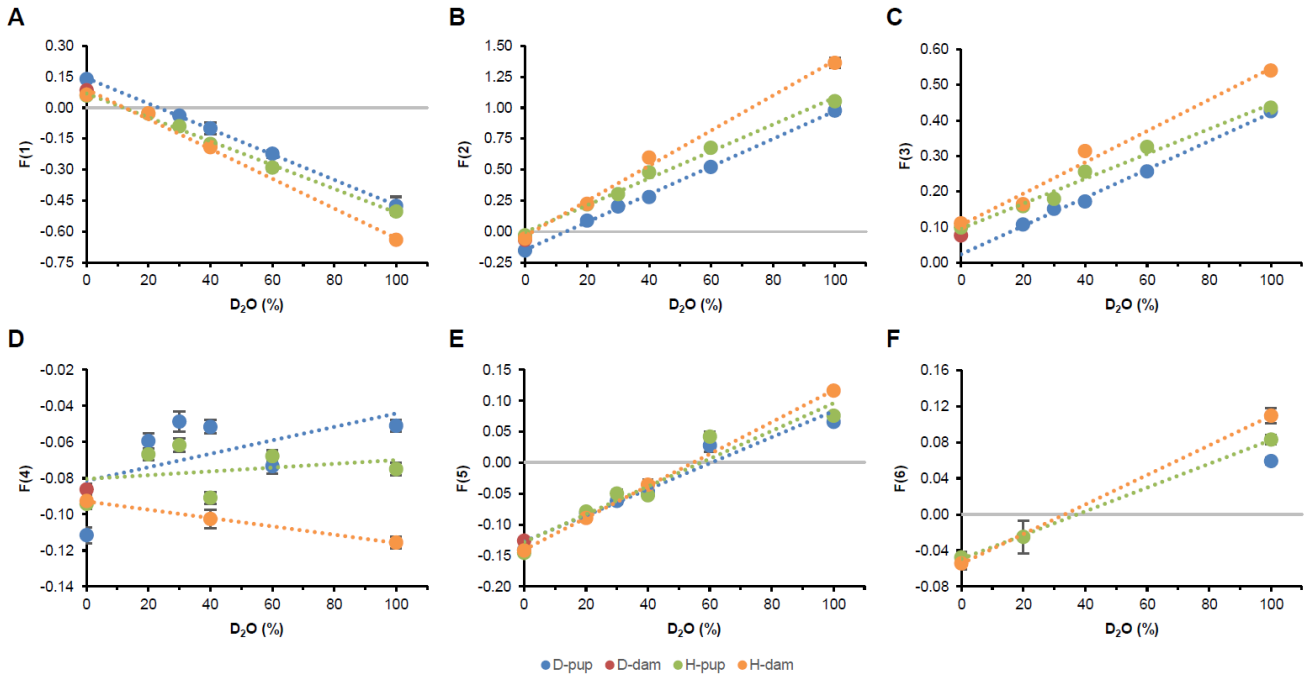



**Figure 3: Structure-factor amplitudes with assigned phases versus %$D_2O$.**

Symbols represent experimental replicates and lines the linear dependence of F(*h*) on %$D_2O$. $\delta$F is calculated by using $\delta F = F * (0.5 * \delta I/I)$, where $\delta$I is the standard deviation in fitting backgrounds and intensities of the Bragg orders (Magicplot Systems, LLC; https://magicplot.com/).

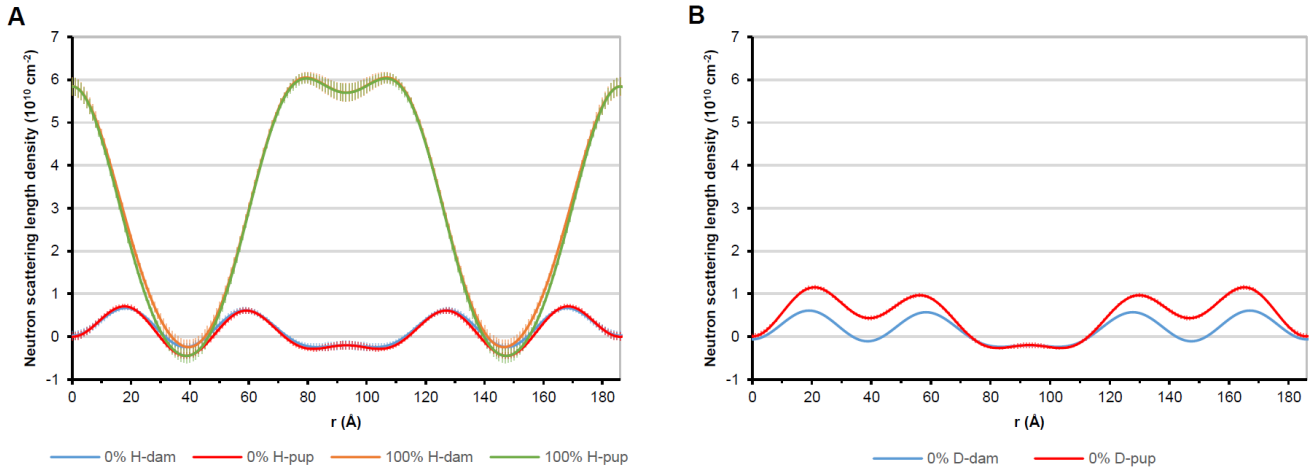



**Figure 4: Scattering density profiles of myelin from pups and dams.**

The profiles were calculated using the interpolated structure amplitudes (for *h*=1–5) based on the linear regression shown in Fig. 3. The origin (r=0 Å) corresponds to the cytoplasmic boundary between membrane pairs. The profiles were scaled such that in 0% and 100% $D_2O$, the absolute density at the extracellular apposition was equal to the neutron scattering densities of $H_2O$ and $D_2O$ containing volume fractions of protein of 0.14, and in the center of the bilayer, a volume fraction of protein of 0.09 (Kirschner et al., 1975). **(A)** H-dam vs H-pup at 0% and at 100% $D_2O$. **(B)** D-pup vs. D-dam at 0% $D_2O$. (Data for D-dam at 100% $D_2O$-PBS was not collected owing to time constraints on the beamline.)

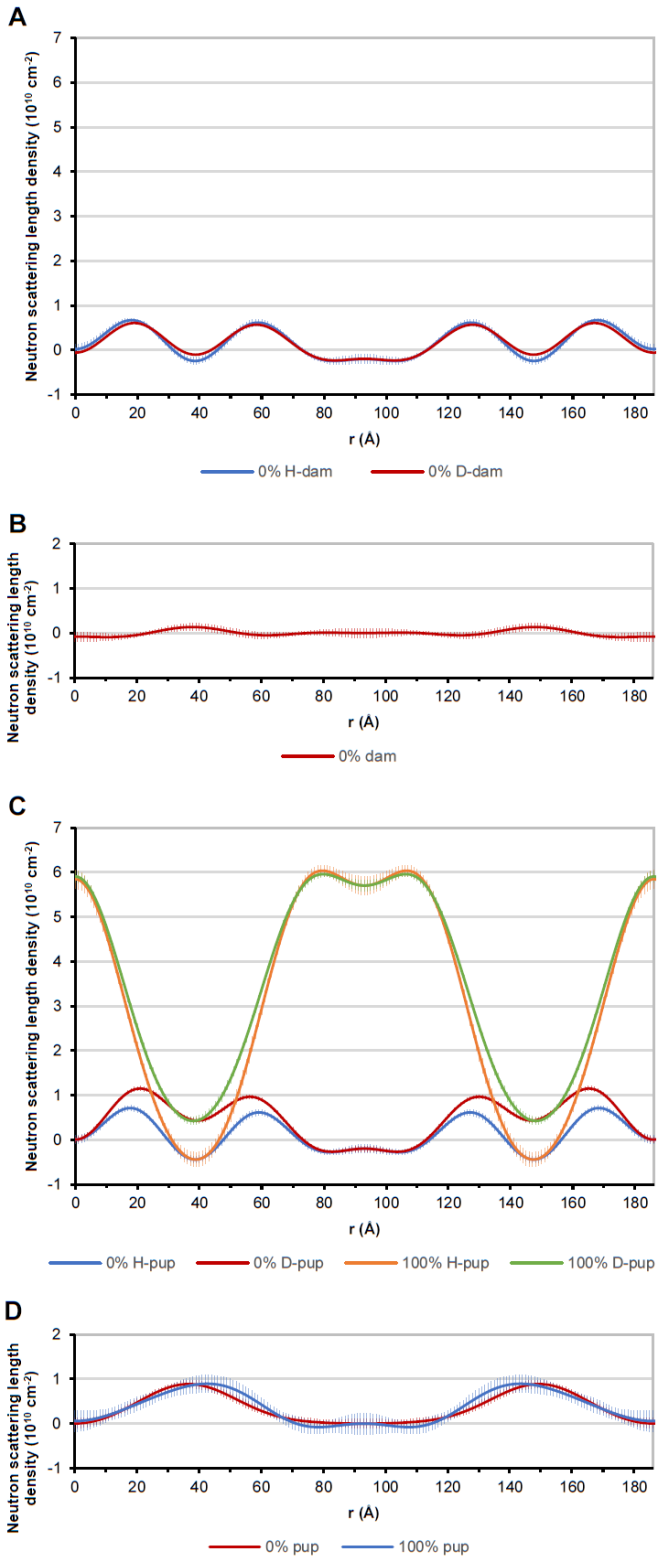
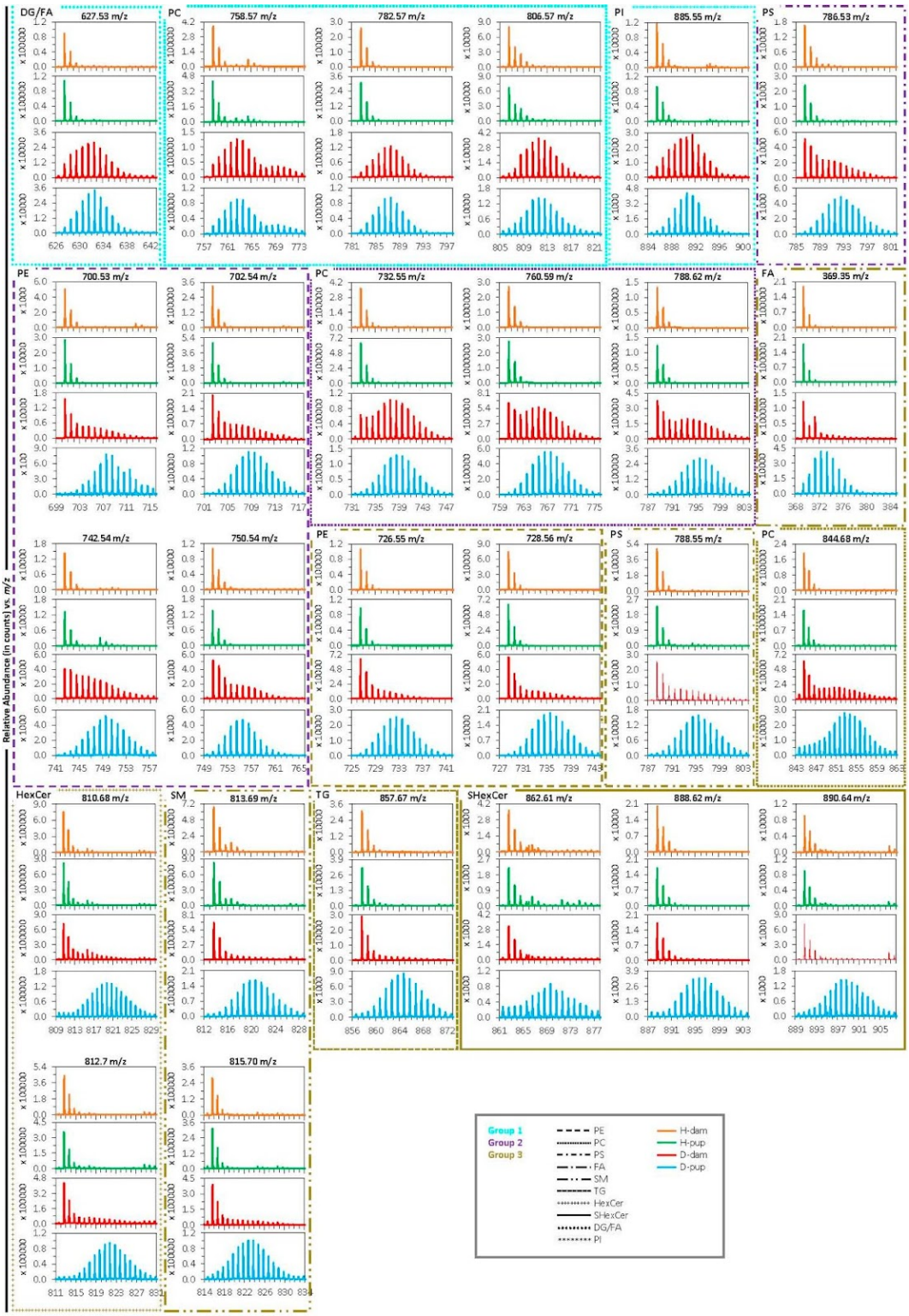



**Figure 5: Scattering density profiles and difference profiles of myelin from D-mice vs. H-mice, all at the same y-scale.**

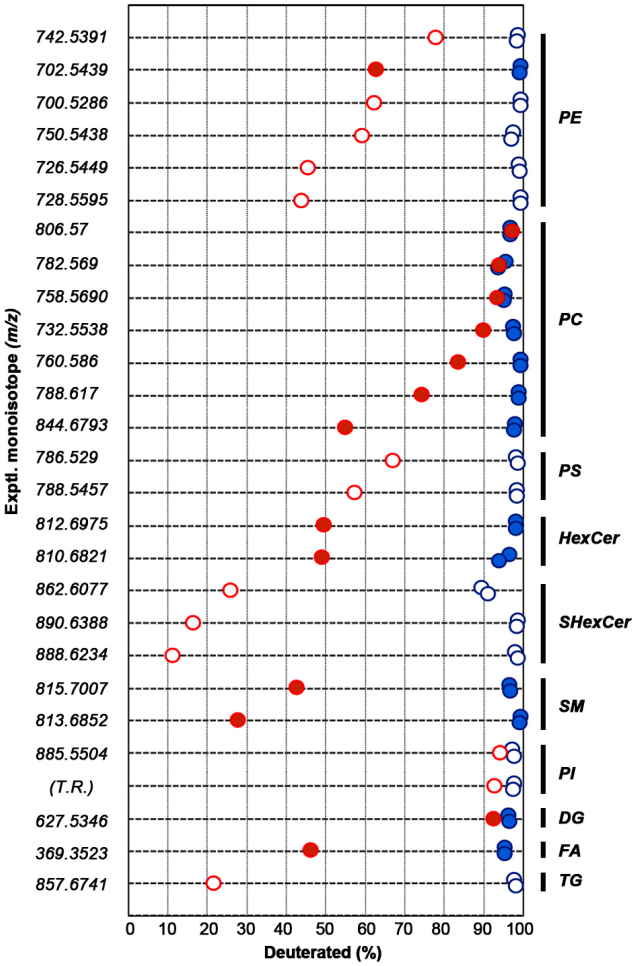
The profiles were calculated using the interpolated structure amplitudes (for h=1–5) based on the linear regression shown in Fig. 3**.** The origin (r=0 Å) corresponds to the cytoplasmic boundary between membrane pairs. The profiles were scaled as described in Fig. 4 and text. **(A)** Scattering density profiles for H-dam and D-dam in 0% $D_2O$-PBS. **(B)** Difference profile between D-dam and H-dam. (For an expanded y-scale, see Suppl. Fig. S9). **(C)** Scattering density profiles for H-pup and D-pup in 0% and 100% $D_2O$-PBS. **(D)** Difference profiles for (D-pup) – (H-pup) in 0% (*red*) and 100% (*blue*) $D_2O$-PBS. (For an expanded y-scale for 0% $D_2O$-PBS, see Suppl. Fig. S9.) Comparison between H-pup and D-pup in the series of solutions of varying contrast showed this consistent difference (Suppl. Fig. S5). Error bars for the difference profile ($\Delta = D - H$) were calculated from: $\Delta = [(\ D)^2 + (\ H)^2]^{½}$, where D and H are the errors at each position in the neutron scattering density profile.

**Figure 6. Mass spectrometry of lipids isolated from mouse trigeminal nerves.**

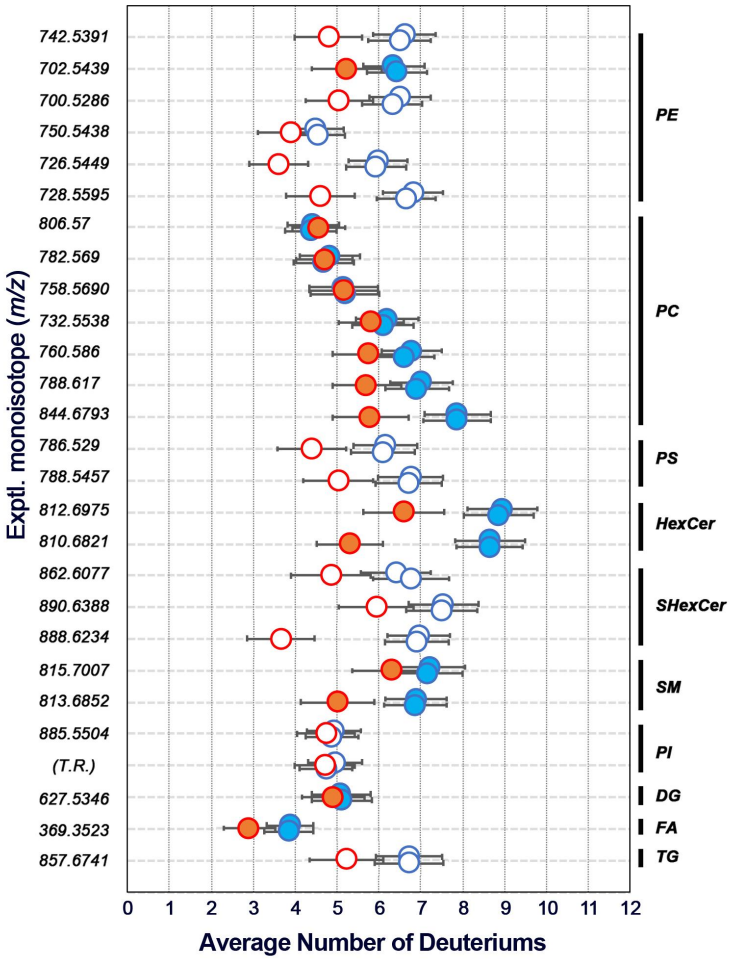



Each vertical set of four spectra (or "panel") corresponds to a different lipid, and shows (top to bottom) the mass isotope distribution as relative abundance vs. *m/z* for H-dam, H-pup, D-dam, and D-pup. For undeuterated samples, the peaks correspond to natural abundance isotopes; and for deuterated samples, the series of peaks at increasing *m/z values* correspond to increasing incorporation of D. The panels are grouped by the extent of deuteration for D-dam: high (bounded by blue), intermediate (bounded by purple), or low (bounded by bilious or olive green). Within each group of panels, lipids of the same species but different values of m/z are enclosed by a box distinguished by a particular line type (see key) and are identified in the upper left corner of the box by an abbreviation.

**Figure 7. Comparison of deuteration levels of lipids in D-dam (red) and D-pups (blue).**




These results are based on mass spectrometry of lipids in negative and positive ion modes, as analyzed using MassWorks (Cerno Bioscience, LasVegas, NV 89144). Of the 89 experimental monoisotopes that we determined using MS-LAMP (Sabareesh, Singh, 2013), 27 (y-axis) corresponded to m/z values that have been identified as specific lipid species in mouse or rat neural tissue and in curated databases (see references cited in Materials & Methods). For each monoisotope value, the percent deuteration levels (x-axis)—calculated by summing the relative percentages of the deuterium isotopologues—are indicated for D-dam (CF0) and D-pups (CF1 and CF4). Seven different species of lipids plus three different species of acylglycerols were identified that corresponded to the *m/z* values: PE (6 sets of data), PC (7 sets), PS (2 sets), HexCer (2 sets), SHexCer (3 sets), SM (2 sets), and PI (1 set, and one technical replicate); and acylglycerols TG (1 set), DG (1 set) and FA (1 set). For each set, the red circle is the deuterated mother, and the blue circles are her pups CF1 and CF4; and fIlled circles indicate the results corresponding to measurements from positive ion mode spectra. T.R. denotes technical replicate (see also Fig. 8).

**Figure 8. Average number of deuteriums per lipid species in D-dam (red) and D-pups (blue).**

The average number of deuteriums for each lipid species was determined by calculating the weighted average of the numbers of deuteriums incorporated for the corresponding D-dam and D-pups. The error bars are standard errors calculated from the weighted standard deviations (Heckert, Filliben, 2003).