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Quantification and Localisation of New Brain Lipid Synthesis Using Deuterium Oxide and High Resolution Mass Spectrometry

Catherine Zhang¹ | Jesse A. Michael² | Jonathan D. Teo¹ | Huitong Song¹ | Mika T. Westerhausen² | Shadrack M. Mutuku² | Shane R. Ellis² | Anthony S. Don¹ 

¹School of Medical Sciences, Charles Perkins Centre, and Brain and Mind Centre, The University of Sydney, Camperdown, New South Wales, Australia |

²Molecular Horizons and School of Science, The University of Wollongong, Wollongong, New South Wales, Australia

Correspondence: Anthony S. Don (anthony.don@sydney.edu.au)

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ABSTRACT

Myelin is the lipid-rich membrane that surrounds neuronal axons and is essential for neurological function in vertebrates. The development of therapeutics that stimulate myelin repair to treat demyelinating disorders such as multiple sclerosis is hampered by the inability to distinguish newly synthesised from pre-existing myelin. This study aimed to develop a method to quantify and localise new myelin lipid synthesis in the mouse brain. Deuterium oxide was administered for two weeks in the drinking water of mice fed normal chow, chow containing the demyelinating toxin cuprizone, or during spontaneous remyelination following cuprizone withdrawal. Liquid chromatography-tandem mass spectrometry and mass spectrometry imaging were used to quantify and localise the newly synthesised, deuterated lipids. While most glycerophospholipids were constitutively deuterated, deuteration of myelin-enriched sulfatides, hexosylceramides, and phosphatidylethanolamine plasmalogens was only apparent during remyelination. Most deuterium atoms were found in the fatty acyl chains, indicative of de novo lipid synthesis. Deuterated hexosylceramide and phosphatidylethanolamine plasmalogen species were localised primarily to the corpus callosum, the white matter tract that is most heavily affected by cuprizone. The method described herein provides the means to quantify and spatially profile dynamic lipid synthesis across diverse biological contexts, including understanding myelin homeostasis and preclinical evaluation of remyelinating therapeutics.

1 | Introduction

Myelin is a lipid-rich membrane produced by specialised cells called oligodendrocytes in the central nervous system, and Schwann cells in the peripheral nervous system. It is wrapped in a spiral fashion around neuronal axons and is required for the rapid conduction of electrochemical signals along axons [1]. Demyelinating diseases such as multiple sclerosis (MS) have debilitating consequences, including vision loss, muscle weakness, and cognitive dysfunction [2]. Current, clinically approved

MS therapeutics are effective at preventing immune-mediated demyelination; however, remyelination is limited, preventing functional recovery. A stronger understanding of factors that modulate remyelination and the development of pro-myelinating therapeutics will improve outcomes for people with demyelinating diseases [3].

Assessing remyelination in vivo is challenged by the inability to identify and quantify newly formed myelin. Measurements of myelin density and thickness using histological staining

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and electron microscopy quantify total myelin content at a given point in time and cannot differentiate newly formed from pre-existing myelin, yet are widely used to assess remyelination [4, 5]. Magnetic resonance imaging can be used to visualise myelin content in living organisms, but it cannot distinguish new from pre-existing myelin. Transgenic mice expressing a fluorescent protein in newly formed oligodendrocytes provide a surrogate measure of new myelin [6] and have been used to follow remyelination in vivo. [4, 7] Although useful, this approach is semi-quantitative, does not quantify actual myelin constituents, and assesses myelin synthesis only by newly formed (as distinct from existing) oligodendrocytes. Additionally, this approach requires complex transgenic models.

Myelin is 70%–80% lipid (dry weight), of which 20%–25% is hexosylceramide (HexCer) and 5% is hexosylceramide-sulfate (sulfatide, SHexCer) [8, 9]. In the central nervous system, over 99% of HexCer and SHexCer are galactosylceramides [10, 11]. These lipids are synthesised exclusively by oligodendrocytes and hence unique to myelin [12–14]. Myelin is also enriched in cholesterol and certain phosphatidylethanolamine plasmalogens (PE-P) [9, 15]. Developments in liquid chromatography-tandem mass spectrometry (LC-MS/MS) over the last two decades have enabled high-throughput analysis and quantification of hundreds of lipids in any given sample. Added to this, mass spectrometry imaging (MSI) now permits the localisation of lipids with low to submicron resolution [16–18]. However, conventional lipidomic analysis and MSI do not discriminate between newly synthesised and pre-existing lipids.

Deuterium oxide ($^2\text{H}_2\text{O}$) administration provides a means to follow new lipid synthesis in a defined time frame through metabolic incorporation of deuterium (^2H) during endogenous lipid synthesis [19]. $^2\text{H}_2\text{O}$ can be administered by mixing with drinking water, rapidly equilibrates with body fluid, and is safe at levels up to 30% in the drinking water of mice [20–22]. During *de novo* lipogenesis, ^2H is incorporated into fatty acid chains through ^2H -labelled acetyl-coenzyme A (CoA) and malonyl-CoA and into the glycerol backbones of triglycerides during glycolysis and glyceroneogenesis [23, 24]. ^2H incorporation into fatty acids can also occur through enzyme-catalysed hydrogen-deuterium exchange from ^2H -labelled nicotinamide adenine dinucleotide phosphate (NADPH) [24]. Early studies used $^2\text{H}_2\text{O}$ in combination with gas chromatography-mass spectrometry (GC-MS) to investigate the turnover of specific fatty acids, cholesterol, and triglycerides in animals and humans [25]. Using pre-fractionation of lipid classes coupled to GC-MS analysis of ^2H -labelled palmitate, Ando et al. demonstrated that myelin cholesterol and galactosylceramide are turned over much slower than glycerophospholipids, and that their turnover is affected by age [26]. More recently, Goh et al. applied $^2\text{H}_2\text{O}$ in combination with LC-MS/MS lipidomic analysis to determine the fractional synthesis of approximately 100 lipids from 13 functional classes in cultured cells. [27] This approach was later adapted by Baumann et al., who administered $^2\text{H}_2\text{O}$ to pregnant mouse dams and compared the levels of deuteration for 26 lipids in developing versus adult neural tissue using LC-MS [28].

In this study, we demonstrate that $^2\text{H}_2\text{O}$ administration in the drinking water can be used in combination with LC-MS/MS

and MSI to quantify and visualise new myelin synthesis in a mouse model of toxin-induced demyelination. Cuprizone is a copper-chelating toxin that induces oligodendrocyte death and demyelination in mice [29]. Spontaneous remyelination occurs within two weeks after cuprizone withdrawal [30]. LC-MS/MS analysis of corpus callosum tissue revealed consistently high levels of deuteration in most glycerophospholipids, indicative of continuous turnover. In contrast, significant deuteration of myelin-enriched HexCer, SHexCer, and PE-P species was only apparent during remyelination following cuprizone withdrawal. MSI revealed localisation of these deuterated lipids to major white matter tracts, particularly the corpus callosum. This approach can be broadly applied to monitor and quantify specific, localised lipid metabolism events in vivo.

2 | Results and Discussion

2.1 | Quantification of Deuterated Lipids in the Corpus Callosum

To quantify new lipid synthesis during demyelination and remyelination, mice were administered 25% $^2\text{H}_2\text{O}$ in their drinking water for the final two weeks of a five-week cuprizone diet (Cpz), or for two weeks after removal of cuprizone from the diet (Rem) (Figure 1a). A control group received only normal mouse chow and 25% $^2\text{H}_2\text{O}$ for two weeks (Ctrl) to measure baseline lipid synthesis. As expected, cuprizone feeding caused significant myelin loss in the corpus callosum, and spontaneous remyelination was apparent two weeks after cuprizone withdrawal (Figure 1b). Remyelination was more notable in the posterior compared to the anterior corpus callosum.

The workflow for quantification of deuterated lipids is shown in Figure 2a. Lipids were extracted from dissected corpus callosum tissue using a two-phase methyl-*tert*-butyl-ether (MTBE)/methanol/water protocol, [31] and subjected to LC-MS/MS analysis with data-dependent acquisition on a Q Exactive HF-X quadrupole-Orbitrap mass spectrometer. Lipid identifications were assigned to 171 LC-MS/MS peaks, comprising the most abundant lipid species in each of 16 lipid classes, based on MS/MS spectra in lipid extracts from mice administered drinking water without $^2\text{H}_2\text{O}$ (Table S1). LC-MS/MS peaks corresponding to deuterated isotopologues ($\text{M}+1$ – $\text{M}+20$) were then identified in corpus callosum lipid extracts from mice administered $^2\text{H}_2\text{O}$, using a combination of accurate precursor m/z (10 ppm mass tolerance) and elution time within 0.1 min of the non-deuterated monoisotope ($\text{M}+0$) (Figure 2b). To eliminate the effects of naturally occurring heavy isotopes on the quantification of deuterated isotopologues, the isotope correction algorithm IsoCor was applied to correct the peak areas based on the natural abundance of heavy isotopes [32]. Lipids were considered to show quantifiable deuteration if (i) the abundance of deuterated isotopologues relative to the $\text{M}+0$ peak (after isotope correction) was below 5% in the $^1\text{H}_2\text{O}$ -only control samples; and (ii) four consecutive isotopologue peaks were obtained in a minimum of three out of four samples in any of the three groups receiving $^2\text{H}_2\text{O}$ (Figure 2c,d). The complete lipidomic dataset containing the amount of each lipid isotopologue is presented in Table S2.

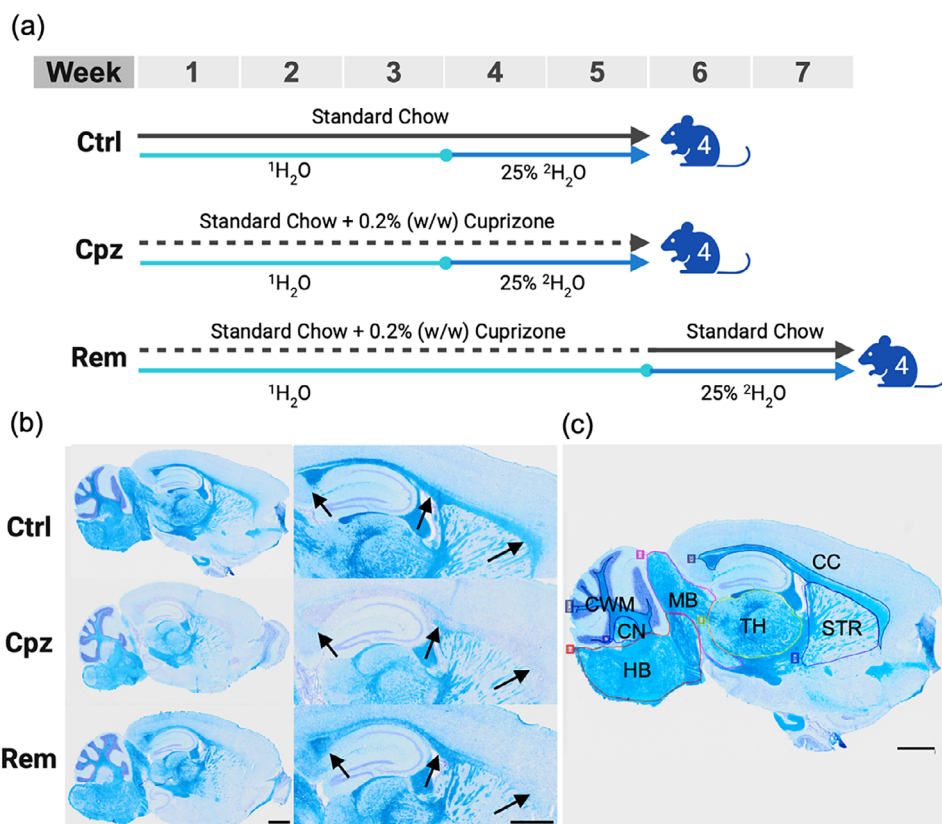


FIGURE 1 | Experimental design. (a) Schematic of the experimental design. Mice were provided 25% $^2\text{H}_2\text{O}$ in their drinking water ($^1\text{H}_2\text{O}$) for the final two weeks of a five-week cuprizone diet (Cpz, $n = 4$), or for two weeks after removal of cuprizone from the diet (Rem, $n = 4$). The control group received only standard chow and 25% $^2\text{H}_2\text{O}$ for the final two weeks (Ctrl, $n = 4$). An additional group of mice (not shown) was provided $^1\text{H}_2\text{O}$ throughout the entire duration of each diet ($n = 1$ each for Ctrl and Cpz, $n = 2$ for Rem). (b) Representative images of Luxol fast blue staining of myelin in sagittal brain sections from each experimental group. Cell nuclei are counterstained with cresyl violet in purple. Scale bar: 1000 μm . Cuprizone induced prominent demyelination in the corpus callosum (indicated by arrowheads), and remyelination was apparent two weeks after cuprizone withdrawal. (c) Annotation of brain regions. From left to right: cerebellar white matter (CWM), cerebellar nuclei (CN), hindbrain (HB), midbrain (MB), thalamus (TH), corpus callosum (CC), and striatum (STR).

2.2 | Identification of Lipid Biomarkers of Myelination

Applying these criteria, deuteration was quantified for 112 of the 171 identified lipids (Figure 3a). Robust deuteration above 25% was observed for 60 lipids in the corpus callosum of the chow-fed Ctrl mice, indicative of constitutive turnover in the two-week $^2\text{H}_2\text{O}$ administration period. These constitutively synthesised lipids were primarily comprised of phosphatidylcholine (PC, all twelve species), phosphatidylinositol (PI, all eight species), diacylglycerol (DG, five of six species), and triacylglycerol (TG, seven of seven species). In contrast, the myelin-enriched lipids, including cholesterol, HexCer (all thirteen species), SHexCer (all six species), and most very-long chain sphingomyelins (SM, ten of thirteen species), showed minimal or no deuteration under basal conditions. This is consistent with the prior observation that turnover of lipids in myelin is slower for cholesterol and HexCer relative to PC and PE [26]. Interestingly, seven of fourteen phosphatidylserine (PS) species were minimally deuterated under basal conditions. These minimally deuterated PS species contained an 18:1 fatty acyl (FA) group paired with a very long-chain (≥ 20 -carbon) saturated/monounsaturated fatty acid, a composition uniquely found in oligodendrocytes [33]. Hence,

the observed differences in baseline lipid deuteration likely reflect distinct cellular origins within the corpus callosum, with the more stable, long-lived lipids representing those produced by oligodendrocytes, and the rapid-turnover lipids representing those found in other neural cells, specifically neurons, astrocytes, and microglia.

The effect of cuprizone intoxication and withdrawal on lipid deuteration was assessed by a two-sample independent t -test for lipids with quantifiable deuteration in two conditions, and one-way ANOVA for lipids with quantifiable deuteration in all three conditions, adjusting p -values for false discovery rate (Table S3). Cuprizone feeding significantly affected the deuterated proportion of 60 lipids (32 increased and 28 decreased). Consistent with the selective toxicity of cuprizone to mature oligodendrocytes and associated loss of myelin [34], the lipids showing decreased deuteration were mostly HexCer, PE-P, and cholesterol. The decrease in the deuterated proportion of these myelin-enriched lipids was accompanied by a decrease in the absolute abundance of their non-deuterated monoisotopes and almost complete absence of the deuterated form (Figure 3b–d). This reflects the loss of lipid that was present prior to $^2\text{H}_2\text{O}$ administration and the lack of new lipid synthesis

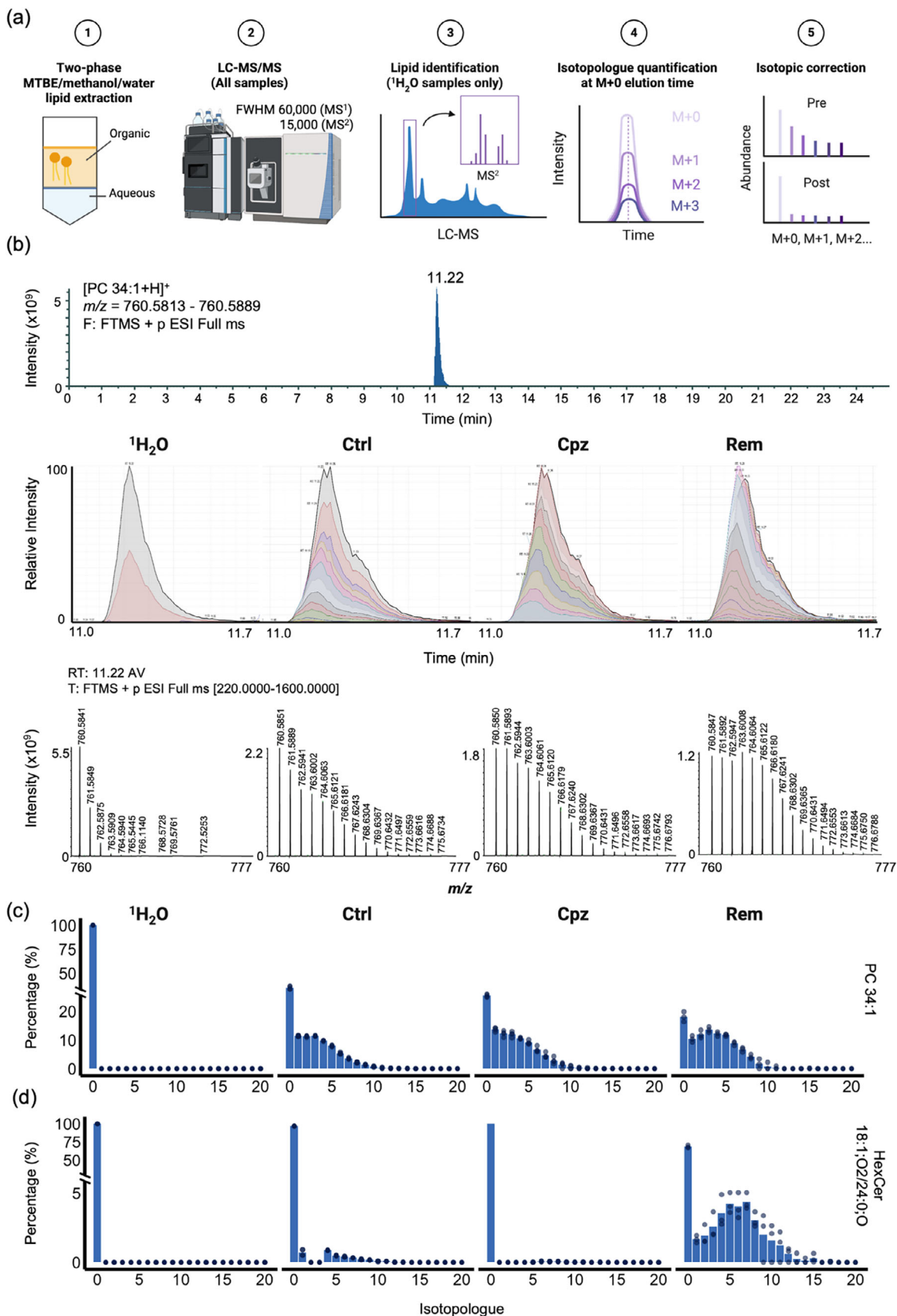


FIGURE 2 | Quantification of deuterated isotopologues. (a) Summary workflow for quantification of deuterated isotopologues. LC-MS/MS data was acquired from corpus callosum lipid extracts using a Q Exactive HF-X mass spectrometer with full width at half maximum (FWHM) mass resolution set to 60,000 (MS¹) and 15,000 (MS²). A list of the most abundant lipids from mice administered only drinking water (¹H₂O control) was constructed based on MS/MS spectra and HPLC elution times. Isotopologues of each lipid were then quantified using the integrated peak area of chromatograms

during cuprizone intoxication. Lipids that showed an increase in proportional deuteration during cuprizone feeding consisted of the more ubiquitous glycerophospholipids along with SM and TG (Figure 3a). Compared to basal conditions, these lipids were synthesised at a similar or higher rate during cuprizone intoxication (Figure 3e–g), consistent with prior findings for cuprizone- and lysolecithin-induced demyelination [35, 36]. Activation and proliferation of astrocytes and microglia are prominent features of the acute cuprizone model [37, 38], and increased mRNA expression of Sphingomyelin Synthase 1 during demyelination has been observed in both cell types [36]. Cuprizone intoxication also induces proliferation of oligodendrocyte precursor cells to replace the lost mature oligodendrocytes [30, 37]. Proliferation of these glial cells probably accounts for the observed increase in synthesis of particular lipids during cuprizone administration.

The withdrawal of cuprizone significantly altered the level of deuteration for 87 lipids and revealed a subset of myelination marker lipids characterised by an absence of deuteration during cuprizone feeding, and robust induction of deuteration during remyelination to levels greater than baseline (Figure 3a). This group encompassed all the quantified HexCer and SHexCer, five of the six PE-P, six PS, three lysophosphatidylethanolamine (LPE), and one each of lysophosphatidylcholine (LPC) and lysophosphatidylserine (LPS). The increase in percentage deuteration of these lipids during remyelination was due to a large increase in the abundance of the deuterated lipid form (Figure 3b,c,h,i), indicating that de novo synthesis of these lipids supports myelin repair. Previous studies reported that levels of HexCer and SHexCer only show partial recovery during spontaneous remyelination, raising the concern that the lipid composition of remyelinated fibres does not recapitulate that of intact myelin [36, 39]. However, the pronounced acceleration in myelin lipid synthesis during remyelination observed herein suggests that a longer time frame may be needed for complete restoration of myelin, and determining rates of lipid synthesis over time will offer more detailed insights into remyelination dynamics.

Lysophospholipids are deacetylated forms of phospholipids generated from the enzymatic cleavage of ester bonds by phospholipase A1 and A2 enzymes [40, 41]. Thus, the increase in deuterated lysophospholipids during remyelination likely stemmed from the hydrolysis of newly synthesised, deuterated glycerophospholipids. These species can be re-incorporated into membrane lipids through the Lands' cycle [41] or serve as lipid mediators to modulate inflammation to support local remyelination [35].

Similar to other major myelin constituents, cholesterol synthesis was significantly increased during remyelination compared to

demyelination (Figure 3d; total deuterated); however, the rate of synthesis remained higher under basal conditions, consistent with previous findings that cholesterol synthesis is rate-limiting for myelination [42]. Our estimate that 2.4% of the cholesterol pool is deuterated after two weeks of $^2\text{H}_2\text{O}$ administration is consistent with a previous study [26], in which the half-replacement time of cholesterol in adult mice administered 30% $^2\text{H}_2\text{O}$ was estimated at 359 days. During demyelination, cholesterol released from degenerating myelin is taken up by phagocytic microglia and stored as cholesteryl esters, which can subsequently be reverse transported during remyelination [43, 44]. However, whether myelin repair after acute demyelination is driven by cholesterol recycling or de novo synthesis remains a subject of debate [44–46]. As cuprizone withdrawal did not restore the level of pre-existing cholesterol (Figure 3d, total non-deuterated), our results suggest that local synthesis of new cholesterol is a major contributor to myelin repair. The pre-existing cholesterol from damaged myelin could be lost from the central nervous system through a locally compromised blood-brain barrier during early demyelination [47], or actively eliminated through enzymatic conversion into 24S-hydroxycholesterol [46].

2.3 | Deuterium is Incorporated Primarily Into the Lipid Acyl Chains

To determine if lipids were deuterated on the lipid headgroup, fatty acyl chains, or both, deuteration of product ions was assessed for five high abundance precursor ions that underwent fragmentation during data-dependent acquisition. Fragmentation of the M+5 isotopologues of PE P-18:0/18:1 and PE P-16:0/18:1 in negative mode yielded product ions corresponding to the headgroup-derived phosphoethanolamine and dehydroglycerol phosphoethanolamine, 18:1 fatty acyl chain, and neutral loss of the 18:1 acyl chain (Figure 4a,b; Table S4). The dehydroglycerol phosphoethanolamine fragment (m/z 196.0380) allows differentiation of PE-P species, which contain a vinyl ether linkage at the sn-1 position, from mass isomers with an alkyl ether-linked sn-1 acyl chain (PE-O). This dehydroglycerol phosphoethanolamine fragment is produced from PE-P and diacyl-linked PE, but not PE-O [48, 49]. The only isotopologue observed for the PE headgroup was M+1, whereas isotopologues up to M+5 could be found for fragments corresponding to the 18:1 acyl chain and neutral loss of the 18:1 acyl chain, implying that both acyl chains are deuterated. The m/z of the 18:1 acyl chain isotopologues differed as expected according to the number of deuterium atoms, despite a consistent mass offset relative to the expected m/z , suggesting that deuteration occurred primarily via metabolic incorporation of deuterium atoms into the fatty acyl chains.

for lipid ions within 10 ppm mass tolerance of the theoretical m/z for deuterated isotopologues, and within a 0.1 min window of the elution time for the monoisotopic M+0 peak. Finally, peak areas were corrected for the abundance of naturally occurring heavy isotopes. (b) Example mass spectra and chromatograms of non-deuterated and deuterated isotopologues of PC 34:1 from each experimental group. Upper panel: full-MS scan in positive ion mode at m/z 760.5851 $\pm$ 5 ppm from a $^1\text{H}_2\text{O}$ -only sample showing the PC 34:1 peak at 11.2 min. Middle panel: chromatograms of PC 34:1 isotopologues in a sample from each treatment group, showing a similar elution time independent of deuterium incorporation. Colours are used to differentiate the peaks for different isotopologues but do not represent a particular isotopologue per se. Bottom panel: full-MS¹ spectra over the m/z range 760.0 – 777.0 at 11.2 min. (c, d) Relative abundance of individual isotopologues from M+0 to M+20, expressed as a percentage of all isotopologues after isotope correction, for PC 34:1 (c) and HexCer 18:1;O2/24:0;O (d). Bars show the mean value for three (Rem) or four ($^1\text{H}_2\text{O}$, Ctrl, Cpz) mice. Dot points represent biological replicates.

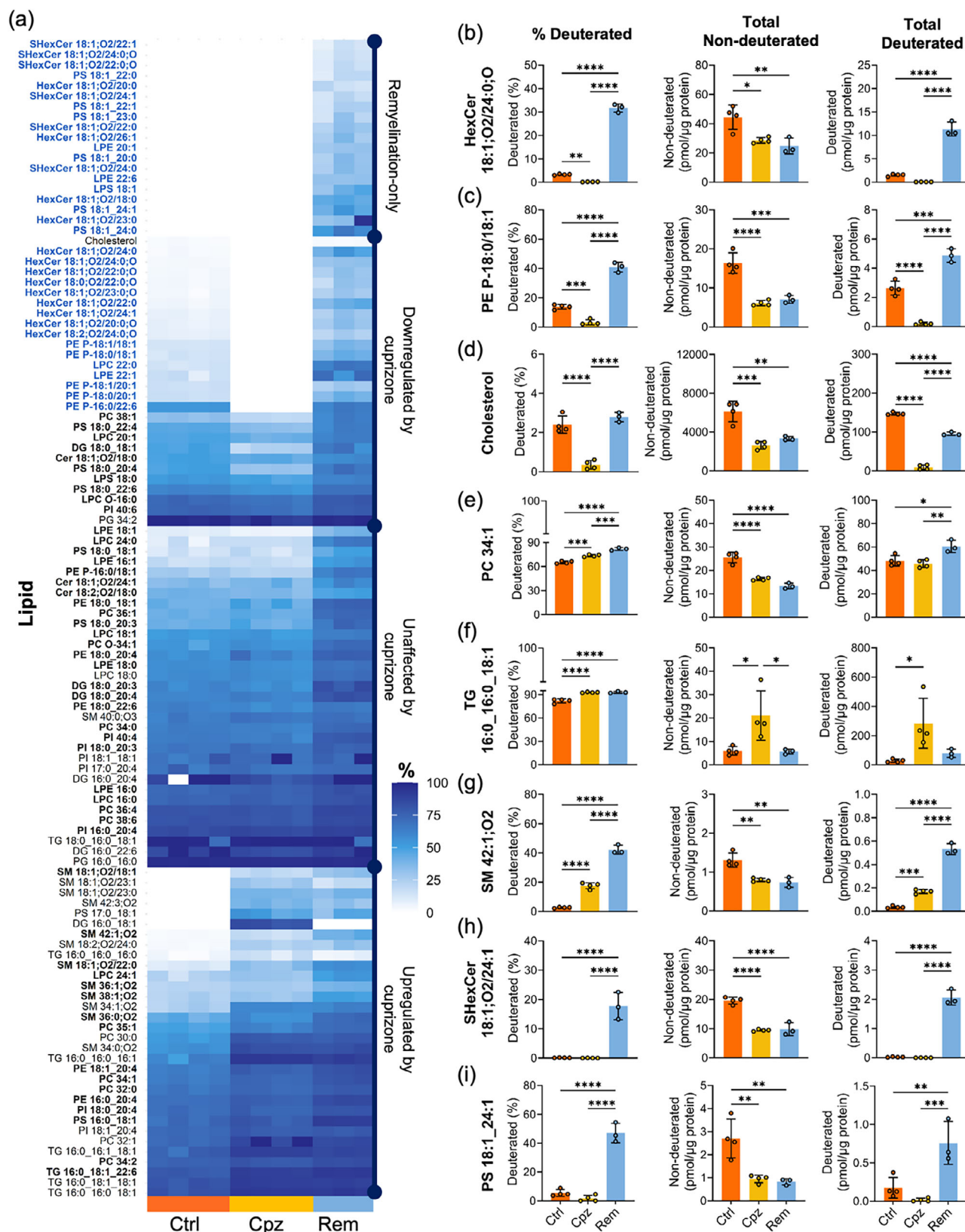


FIGURE 3 | Identification of lipid biomarkers for myelination. (a) Heat map of percentage deuteration for lipids defined as having quantifiable deuteration. Colour scale indicates percentage deuteration. Each column represents one mouse. Data was not acquired for one of the four mice in the remyelination (Rem) group, due to a failed autosampler injection. Lipids with non-quantifiable deuteration in one or two of the experimental conditions were assigned a value of zero. Lipids were grouped into four categories based on changes in deuteration in response to cuprizone treatment and withdrawal. Myelination marker lipids (blue font) were identified based on the absence of deuteration during cuprizone intoxication, and a statistically significant increase in deuteration relative to basal conditions during remyelination. (b-i) Percent deuteration, and total amounts of non-deuterated and deuterated

The relative paucity of deuteration on the PE headgroup may reflect the much greater number of hydrogens in the fatty acyl chains.

Fragmentation of the M+6 isotopologue of [PE 18:0_20:4 -H]⁻ yielded fragments with up to two ²H atoms for the PE headgroup, six for the 18:0 acyl chain, and three for the 20:4 acyl chain (Figure 4c; Table S4). The most abundant isotopologues for the 18:0 acyl chain were M+4 and M+5, whereas M+0 was most abundant for the 20:4 chain, suggesting that the 18:0 chain was newly synthesised during the ²H₂O administration period. In addition, deuteration appears variable for the same fragment in different lipids. Fragmentation of M+6 [SM 42:1;O2 +H]⁺ and M+7 [HexCer 18:1;O2/24:0;O +H]⁺ yielded the full range of possible isotopologues for the 18:1 sphingoid base; however, the most abundant isotopologue of this fragment was M+4 for the SM, and M+0 for the HexCer (Figure 4d,e; Table S4). Fragments corresponding to the choline phosphate headgroup in SM were indicative of both ¹³C and ²H atoms. The mass difference between the 185.0769 fragment and monoisotopic 184.0733 fragment is consistent with a ¹³C atom, while the adjacent peak at *m/z* 186.0779 is most consistent with one ¹³C and one ²H atom. As the resolution of the product ion scan used for this experiment does not resolve between the two isotopes, the detected *m/z* for these choline phosphate fragments most likely represent a mixture of [M+ ¹³C] and [M+ ²H] species.

2.4 | Synthesis of Myelin-Enriched Lipids is Localised to the Corpus Callosum During Remyelination

To map the localisation of newly synthesised lipids during remyelination, mass spectrometry imaging was applied to sagittal brain sections from the same mice as used for the above-described LC-MS/MS analysis. Tissue sections were pre-coated in non-endogenous lipid standards for signal normalisation and quantification before 2,5-dihydroxyacetophenone matrix application. Sections were analysed at 30 μm spatial resolution (pixel raster) using atmospheric pressure matrix-assisted laser desorption/ionisation (MALDI) and plasma post-ionisation on a Bruker timsTOF Pro mass spectrometer (mass resolution 35,000–40,000 FWHM at *m/z* 750) [18]. Due to the dense spectra lacking chromatographic separation of isobaric lipids and isotopologues, representative species [PC 34:1 +H]⁺, [PE P-34:1 +H]⁺, and [HexCer 40:1;O3 +H]⁺ were selected for analysis, as they exhibited minimal isobaric interference for their respective M+0 – M+6 peak clusters (Table S5). Spectral peaks corresponding to the M+1 – M+3 isotopologues of [PC 34:1 +H]⁺ (Figure 5a,b), [PE P-34:1 +H]⁺ (Figure 6a,c), and [HexCer 40:1;O3 +H]⁺ (Figure 6b,d) were observed in all groups, including mice administered only ¹H₂O. Their presence in ¹H₂O control mice is most likely due to naturally occurring ¹³C isotopes. Peaks corresponding to the M+4

– M+6 isotopologues of these lipids were only detected in mice administered 25% ²H₂O.

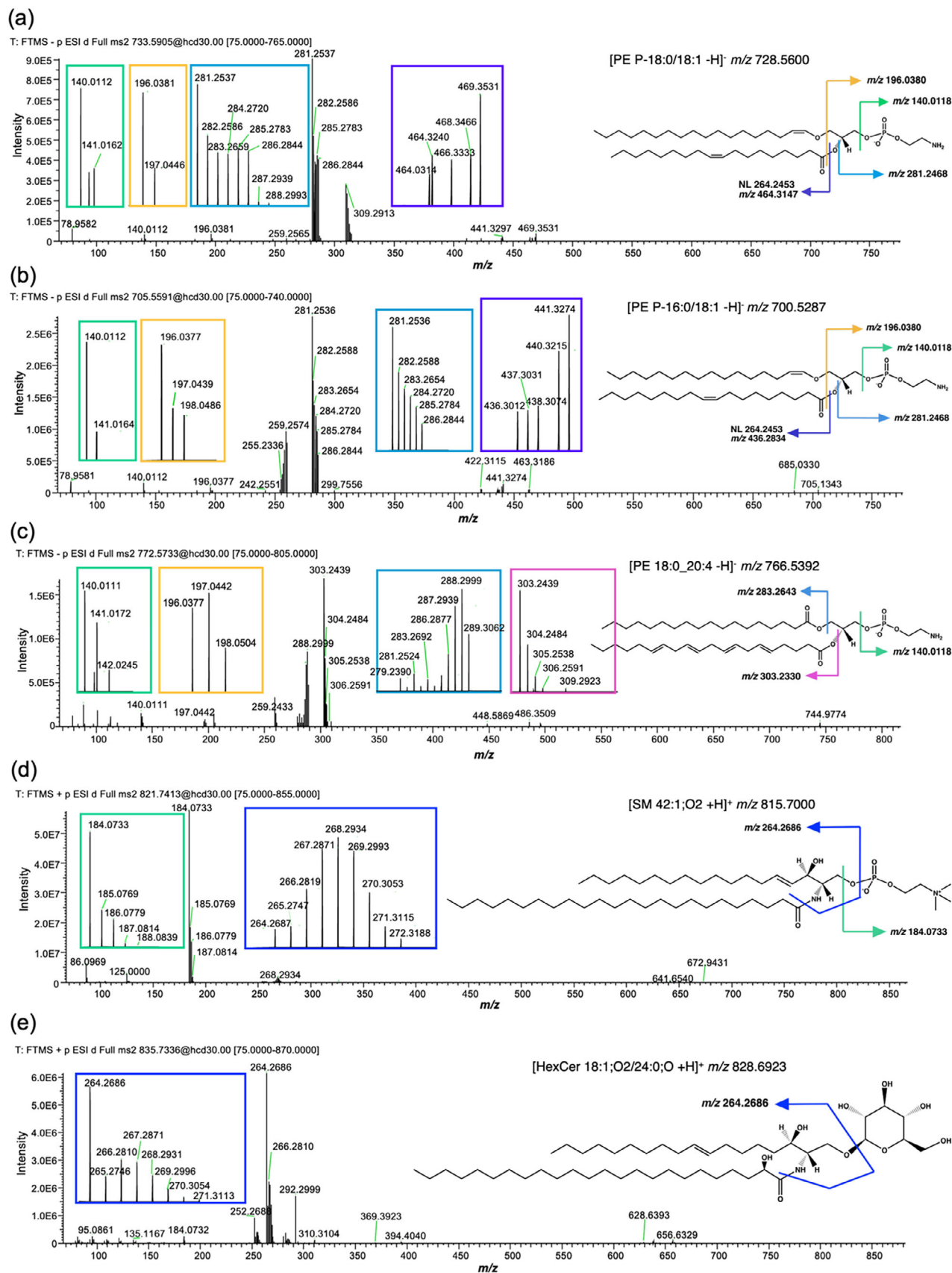
In agreement with prior MSI studies [50, 51], the M+0 isotopologue of PC 34:1 was distributed homogeneously throughout the brain of Ctrl mice (Figure 5c). The M+2 – M+6 isotopologues of PC 34:1 were substantially more abundant in the grey matter regions, suggesting that basal turnover of PC 34:1 is slower in the white matter compared to the grey matter. In contrast, PE P-34:1 and HexCer 40:1;O3 were predominantly localised to the white matter tracts such as the corpus callosum, cerebellar and striatal white matter, and subcortical structures including the midbrain, hindbrain, and thalamus (Figure 6e,f). Consistent with the slow turnover of myelin, signals for the M+4 – M+6 isotopologues of these lipids were substantially lower than those of the monoisotopic species (Figure 6a–d).

To assess changes in pre-existing and newly synthesised lipids during demyelination and remyelination, the abundance of the M+0 and M+6 lipid isotopologues was quantified in each of the annotated brain regions. Cuprizone treatment did not affect the M+0 or M+6 isotopologues of PC 34:1, apart from a localised increase in M+6 within the corpus callosum (Figure 5c,d). This observation aligns with our LC-MS/MS data, indicating continued synthesis of PC 34:1 in this region during demyelination (Figure 3e), likely driven by proliferating glial cells. Following cuprizone withdrawal, the monoisotopic signal for PC 34:1 declined in the corpus callosum and cerebellar nuclei, consistent with the turnover of pre-existing PC 34:1 and its replacement by newly synthesised, deuterated lipid, as evident in increased levels of the M+6 form (Figure 5d).

Cuprizone feeding significantly reduced levels of the PE P-34:1 and HexCer 40:1;O3 monoisotopes in the corpus callosum, and PE P-34:1 in the cerebellar nuclei (Figure 6g,h). This is consistent with histological evidence showing that these regions undergo the most pronounced demyelination in this model (Figure 1b) [29], and therefore exhibit the greatest demand for remyelination. A pronounced enrichment of the M+6 form of these lipids was observed in the corpus callosum during remyelination (Figure 6e,f). Comparing the remyelination to the cuprizone condition, mean levels of the M+6 isotopologues of PE P-34:1 and HexCer 40:1;O3 increased 3.4- and 5.3-fold, respectively, in the corpus callosum, 3.0- and 4.4-fold in the cerebellar nuclei, 2.6- and 2.9-fold in the cerebellar white matter, 1.9- and 3.4-fold in the striatum, and 2.1- and 3.1-fold in the thalamus (Figure 6g,h).

These results demonstrate the capability of combining ²H₂O labelling with MSI to visualise sites of dynamic lipid synthesis, and by extension, new myelin synthesis following cuprizone withdrawal. While remyelination was not apparent in the anterior corpus callosum by histological staining (Figure 1b), MALDI-MSI showed synthesis of new myelin lipids in this region.

deuterated HexCer 18:1;O2/24:0;O (b), PE P-18:0/18:1 (c), free cholesterol (d), PC 34:1 (e), TG 16:0_16:0_18:1 (f), SM 42:1;O2 (g), SHexCer 18:1;O2/24:1 (h), and PS 18:1_24:1 (i). Statistical significance was determined by one-way ANOVA with Tukey's post-test for lipids with quantifiable deuteration in all three conditions, or by two-sample *t*-test for lipids with quantifiable deuteration in two out of three conditions. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. Error bars show standard deviation (*n* = 4 for Ctrl, *n* = 4 for Cpz; *n* = 3 for Rem). *p*-Values were corrected for false discovery rate, and lipids with a significant increase in deuteration during remyelination relative to the basal condition, at *q* < 0.05, are in bold font in part (a).



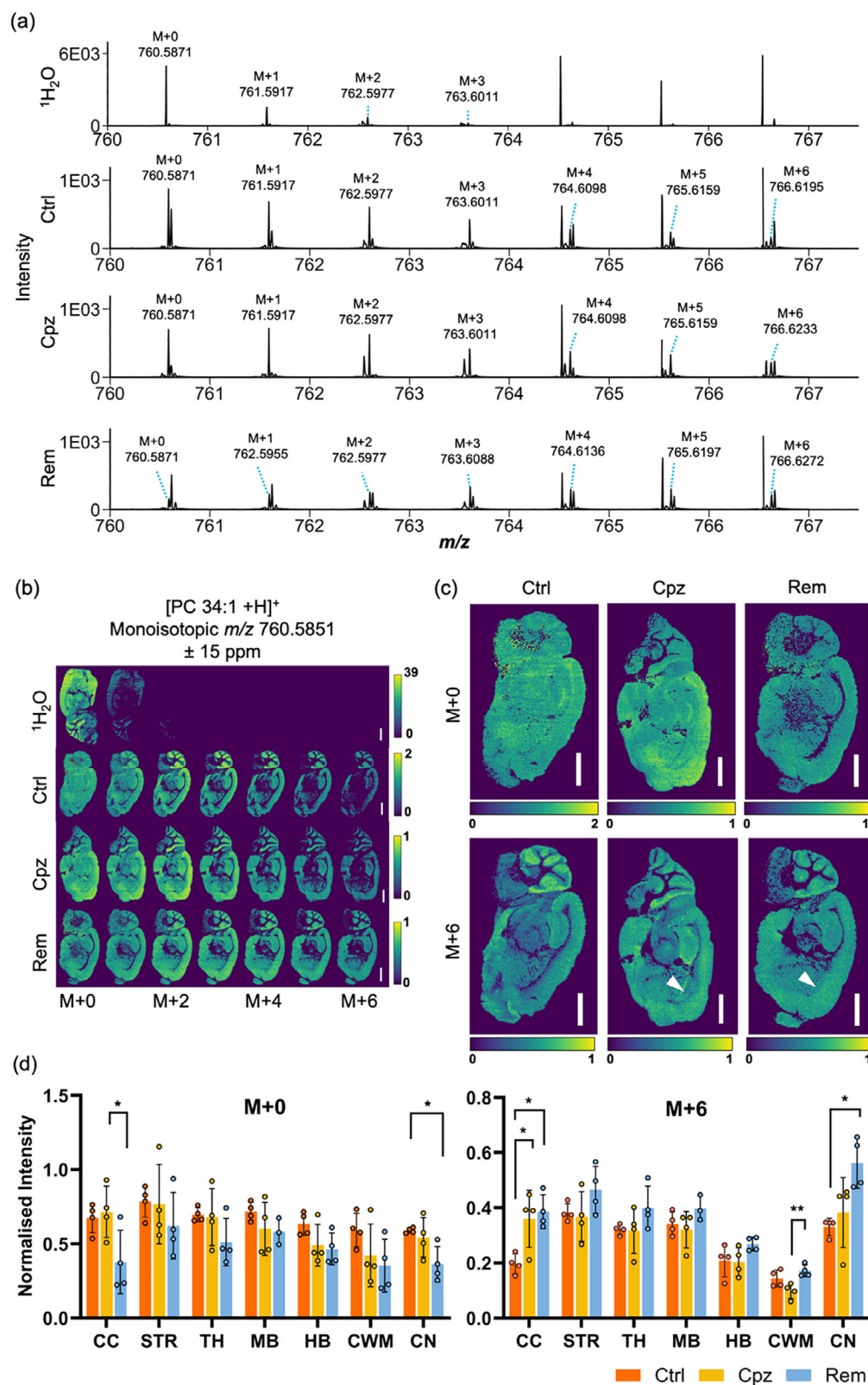


FIGURE 5 | Visualisation of PC 34:1 isotopologues using MALDI-MSI. (a) Full scan MALDI-MS spectra of the corpus callosum from m/z 760.0 – 767.0 showing the presence of deuterated $[PC\ 34:1 + H]^+$. (b) Corresponding ion images of $[PC\ 34:1 + H]^+$ isotopologues from M+0 – M+6 extracted with a ± 15 ppm peak interval, and (c) enlarged images of the M+0 and M+6 isotopologues. The colour scale represents the intensity of the ion of interest normalised to the $[PC\ 33:1[D7] + H]^+$ internal standard on a per-pixel level. One representative image from each experimental group is presented. Scale bar: 2000 μm . Arrowheads denote increased M+6 localisation in the anterior corpus callosum during demyelination and remyelination. (d) Mean normalised intensity of the M+0 and M+6 isotopologue in the corpus callosum (CC), striatum (STR), thalamus (TH), midbrain (MB), hindbrain (HB), cerebellar white matter (CWM), and cerebellar nuclei (CN). Error bars show standard deviation (4 mice per group). Statistical significance was determined by one-way ANOVA with Tukey's post-test for each brain region: * $p < 0.05$, ** $p < 0.01$.

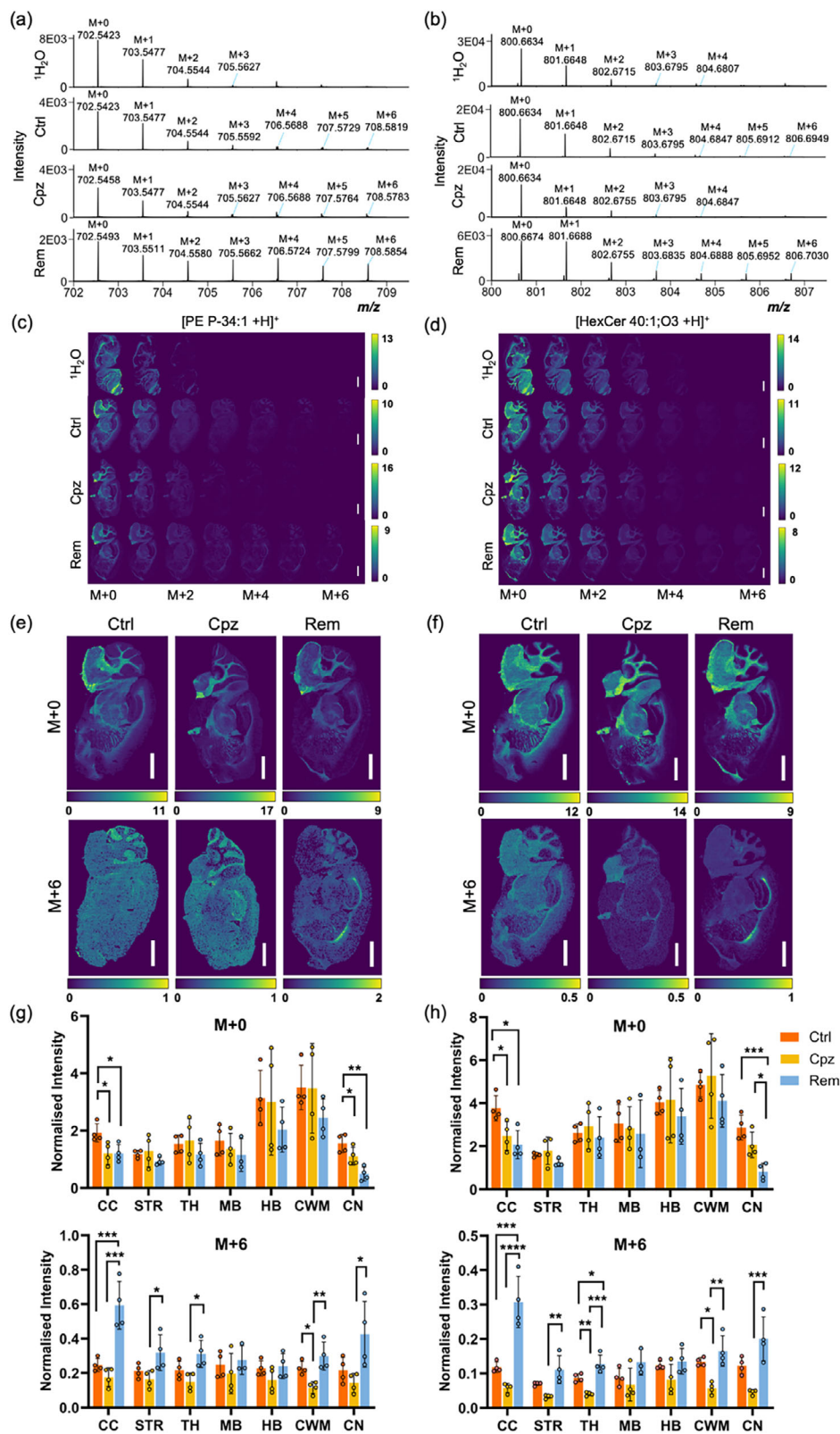


FIGURE 6 | Newly synthesised myelin lipids localise to the corpus callosum during remyelination. (a, b) Full scan MALDI-MS spectra of the corpus callosum from m/z 702.0 – 709.0 (a) and m/z 800.0 – 807.0 (b) showing the presence of deuterated [PE P-34:1 + H] $^+$ and [HexCer 40:1;O3 + H] $^+$, respectively. (c, d) Corresponding ion images of isotopologues from M+0 – M+6 extracted with a $\pm$ 15 ppm peak interval. (e, f) Enlarged images of the M+0 and M+6 isotopologues. Colour scale represents the ion intensity normalised to the [PE 33:1[D7] + H] $^+$ (e) or [HexCer 35:1;O2 + H] $^+$ internal standard. One representative sample from each experimental group is presented. Scale bar: 2000 μm . (g, h) Mean normalised intensity for the M+0 and M+6 isotopologues of PE P-34:1 (g) and HexCer 40:1;O3 (h) in the corpus callosum (CC), striatum (STR), thalamus (TH), midbrain (MB), hindbrain (HB), cerebellar white matter (CWM), and cerebellar nuclei (CN). Error bars show standard deviation (4 mice per group). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 by one-way ANOVA with Tukey's post-test for each brain region.

Since retention of Luxol fast blue in myelin is dependent on its lipophilic interaction with the densely packed myelin sheath [52], these results most likely indicate that remyelination of the posterior corpus callosum is more complete than the anterior corpus callosum at the two-week time point used for our study. We speculate that the deuterated PE P-34:1 and HexCer 40:1;O3 detected in the anterior corpus callosum by MSI have not yet been assembled into dense, compact myelin sheaths that retain Luxol fast blue. Further studies investigating multiple time points after cuprizone withdrawal would shed light on differences in rates of remyelination across different brain regions and whether different lipids are produced during different stages of myelination.

3 | Conclusion

This study demonstrates the use of $^2\text{H}_2\text{O}$ administration in combination with high resolution mass spectrometry to quantify and visualise new lipid synthesis in the mammalian brain. Our approach builds on previous spatial metabolomics studies using stable isotope tracers for central carbon metabolites [53], instead using $^2\text{H}_2\text{O}$ to enable unbiased mapping of lipid metabolism events in living organisms. We applied these methods to demonstrate the differential synthesis rates of brain lipids and specific locations of myelin lipid synthesis following a demyelinating insult. This is important for the discovery and evaluation of remyelinating therapeutics, which are needed to enhance functional recovery and clinical outcomes in people with demyelinating diseases. More broadly, the methods described herein can be applied to many biological settings that require quantification and localisation of small molecule synthesis over a defined timeframe in living organisms.

Supporting Information

The authors have cited additional references within the Supporting Information [54–57].

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Metabolomics Workbench, Study ID ST004338, DOI: [10.21228/M8655C](https://doi.org/10.21228/M8655C); and Zenodo, DOI: [10.5281/zenodo.17504677](https://doi.org/10.5281/zenodo.17504677).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: anie71849-sup-0001-TableS1.xlsx.

Supporting File 2: anie71849-sup-0002-TableS2.xlsx.

Supporting File 3: anie71849-sup-0003-TableS3.xlsx.

Supporting File 4: anie71849-sup-0004-TableS4.xlsx.

Supporting File 5: anie71849-sup-0005-TableS5.xlsx.

Supporting File 6: anie71849-sup-0006-TableS6.xlsx.

Supporting File 7: anie71849-sup-0007-SuppMat.docx