Sphingosine kinase 2 is essential for remyelination following cuprizone intoxication

Running title: SphK2 is essential for remyelination

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Acknowledgements: This research was funded by project grant APP1100626 and Ideas grant APP2002660 from the National Health and Medical Research Council (NHMRC), Australia (A.S.D.); and MS Research Australia grants 18-0477 (J.D.T. and A.S.D.) and 20-0113 (A.S.D.). We gratefully acknowledge subsidised access to the Sydney Mass Spectrometry and Sydney Microscopy and Microanalysis core facilities, and subsidised mouse housing provided by Laboratory Animal Services, University of Sydney.

Word count:

Total: 7330 (including citations and figure legends)

Abstract: 246

Introduction: 759 (including citations)

Materials and methods: 1809 (including citations)

Results: 1628 (including citations)

Discussion: 1823 (including citations)

Figure legends: 1065

Abstract

Therapeutics that promote oligodendrocyte survival and remyelination are needed to restore neurological function in demyelinating diseases. Sphingosine 1-phosphate (S1P) is an essential lipid metabolite that signals through five G-protein coupled receptors. S1P receptor agonists such as Fingolimod are valuable immunosuppressants used to treat multiple sclerosis, and promote oligodendrocyte survival. However, the role for endogenous S1P, synthesized by the enzyme sphingosine kinase 2 (SphK2), in oligodendrocyte survival and myelination has not been established. This study investigated the requirement for SphK2 in oligodendrocyte survival and remyelination using the cuprizone mouse model of acute demyelination, followed by spontaneous remyelination. Oligodendrocyte density did not differ between untreated wild-type (WT) and SphK2 knockout (SphK2$^{-/-}$) mice. However, cuprizone treatment caused significantly greater loss of mature oligodendrocytes in SphK2$^{-/-}$ compared to WT mice. Following cuprizone withdrawal, spontaneous remyelination occurred in WT but not SphK2$^{-/-}$ mice, even though progenitor and mature oligodendrocyte density increased in both genotypes. Levels of cytotoxic sphingosine and ceramide were higher in the corpus callosum of SphK2$^{-/-}$ mice, and in contrast to WT mice, did not decline following cuprizone withdrawal in SphK2$^{-/-}$ mice. We also observed a significant reduction in myelin thickness with ageing in SphK2$^{-/-}$ compared to WT mice. These results provide the first evidence that SphK2, the dominant enzyme catalysing S1P synthesis in the adult brain, is essential for remyelination following a demyelinating insult and myelin maintenance with ageing. We propose that persistently high levels of sphingosine and ceramide, a direct consequence of SphK2 deficiency, may block remyelination.

Keywords

Myelin, remyelination, multiple sclerosis, sphingosine 1-phosphate, sphingosine kinase, S1P, SPHK2

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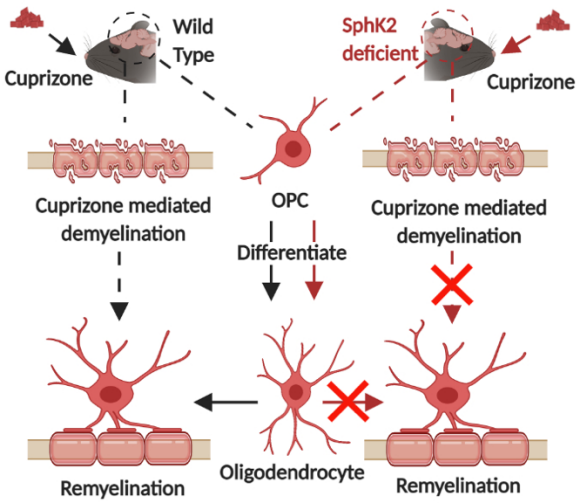

Main Points

- The lipid kinase sphingosine kinase 2 (SphK2) is essential for remyelination following a demyelinating insult (cuprizone).
- SphK2 protects against cuprizone-mediated oligodendrocyte loss.
- SphK2 deficiency leads to thinner myelin with ageing.

Introduction

Myelin is a multi-layered membrane that surrounds neuronal axons and is necessary for rapid and energy-efficient propagation of action potentials (Roth & Nunez, 2016; Simons & Nave, 2016). Myelin is synthesized by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system. Oligodendrocytes and myelin are essential not only for rapid impulse propagation, but also for neuronal bioenergetics and survival (Simons & Nave, 2016).

Immune-mediated degradation of myelin is the cause of multiple sclerosis (MS) (Reich, Lucchinetti, & Calabresi, 2018). Immunosuppressive therapies are generally effective at blunting neuroinflammation and demyelination in relapsing-remitting MS, which is the most common form of the disease. This slows disease progression, however endogenous myelin repair is limited. Relapsing-remitting MS eventually progresses into secondary progressive MS, characterised by irreversible decline. Therapeutics that promote oligodendrocyte survival and remyelination are needed to restore neurological function in MS and other demyelinating diseases (Plemel, Liu, & Yong, 2017; Stangel, Kuhlmann, Matthews, & Kilpatrick, 2017).

Myelin is comprised 70-80% of lipids, and is enriched in the sphingolipids galactosylceramide (a.k.a. cerebroside), sulfatide, and sphingomyelin, which are all synthesized from ceramide (Schmitt, Castelvetri, & Simons, 2015). Alternatively, ceramide can be de-acylated by ceramidases to produce sphingosine, which is phosphorylated by sphingosine kinase 1 (SphK1) or 2 (SphK2) to produce the signalling lipid S1P (Grassi et al., 2019). SphK2 is the dominant isoform in the brain, as S1P levels are ~85% lower in grey matter of SphK2 knockout (SphK2$^{-/-}$) mice (Lei et al., 2017). In accordance with the high levels of sphingolipids in myelin, S1P

levels are 3- to 10-fold higher in white matter compared to grey matter (Couttas et al., 2014; Wang, Kano, Saigusa, & Aoki, 2019).

After its synthesis, S1P is secreted and signals through a family of five G-protein coupled receptors, S1PR1-S1PR5 (Choi & Chun, 2013; Grassi et al., 2019). S1P receptors 1-3 (S1PR1-3) are expressed by most cells and tissues, although S1PR1 expression is particularly high in astrocytes and endothelial cells. S1PR4 expression is restricted to immune cells, whilst S1PR5 is expressed only by mature oligodendrocytes in the CNS (Jaillard et al., 2005; Y. Zhang et al., 2014). *In vitro* cell culture studies have shown that S1P, signalling through S1PR1, stimulates oligodendrocyte progenitor cell (OPC) proliferation (Jung et al., 2007), whereas S1PR5 promotes oligodendrocyte survival (Jaillard et al., 2005).

The MS drug Fingolimod is a sphingosine analogue that, when phosphorylated by SphK2 *in vivo*, becomes a potent agonist of S1P receptors 1, 3, 4, and 5 (Bigaud, Guerini, Billich, Bassilana, & Brinkmann, 2014; Mandala et al., 2002). Its clinical success led to the recent approval of selective S1PR1/S1PR5 agonists Siponimod and Ozanimod for treatment of MS, with other S1PR1 agonists currently in clinical trials (Bigaud et al., 2014; Stepanovska & Huwiler, 2020). Suppression of autoimmunity with this class of drugs is attributed primarily to hyper-activation of S1PR1 receptors on lymph-node resident lymphocytes. This impedes the S1PR1-dependent egress of lymphocytes from lymph nodes into the circulation (Mandala et al., 2002; Matloubian et al., 2004), and is therefore termed “functional antagonism” of S1PR1. In addition to its peripheral effects, fingolimod readily crosses the blood brain barrier, and the effectiveness of the drug in the rodent model of MS, experimental autoimmune encephalitis (EAE), is at-least partly attributed to its action on astrocyte S1PR1 receptors (Choi et al., 2011). Fingolimod also protects against demyelination induced with the oligodendrocyte toxin

cuprizone (H. J. Kim et al., 2011; S. Kim et al., 2018), but does not enhance spontaneous remyelination after cuprizone withdrawal (Alme et al., 2015; Nystad et al., 2020). It remains to be established if endogenous S1P is an important mediator of oligodendrocyte survival and remyelination *in vivo*.

We recently demonstrated that SphK2 deficiency synergises with an amyloidogenic transgene to cause oligodendrocyte loss and demyelination in a mouse model of Alzheimer's disease (Lei et al., 2019). Herein we employed the oligodendrocyte toxin cuprizone to determine if endogenous SphK2 protects against demyelination and/or is necessary for remyelination following a well-established demyelinating insult. Cuprizone administration in the diet is accompanied by acute demyelination attributed to oligodendrocyte death (Benardais et al., 2013; Zhan et al., 2020). Spontaneous remyelination occurs within 2-3 weeks of cuprizone withdrawal, making this an ideal model for investigating factors that regulate oligodendrocyte survival and myelin elaboration in adult mice. We demonstrate that endogenous SphK2 protects against cuprizone-mediated oligodendrocyte loss, and is essential for remyelination following cuprizone withdrawal. We also establish that myelin sheaths are thinner in aged SphK2$^{-/-}$ mice, suggesting that SphK2 is necessary for myelin turnover or biosynthesis in the adult mouse, but not for developmental myelination.

Experimental Methods

Transgenic mice and cuprizone administration

SphK2 knockout mice (SphK2$^{-/-}$) (RRID: IMSR_JAX:019140) were obtained under MTA with Prof Richard Proia, NIDDK, Bethesda, USA and genotyped as described (Mizugishi et al., 2005). SphK2$^{-/-}$ mice were crossed to C57BL/6J mice for more than 10 generations. SphK2$^{-/-}$ and wild-type (WT) control (SphK2$^{+/+}$) mice used in this project were littermates derived from SphK2$^{+/-}$ breeding pairs. Male mice were used for all experiments. Mice were housed in independently-ventilated cages, with food and water provided *ad libitum* and a 12 h light/dark schedule, and weighed three times per week. Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and approved by the University of Sydney animal ethics committee (2017/1284).

Groups of 6-8 mice, at 3 months of age, were randomly allocated to treated (0.2% (w/w) cuprizone in normal chow) or untreated (normal chow) groups. Cuprizone (Sigma Aldrich, #C9012) was incorporated into chow pellets by Specialty Feeds, Australia. Mice were culled by isofluorane inhalation, then perfused transcardially with sterile saline. Brains were removed and halved sagittally. One hemisphere was post-fixed overnight with 4% paraformaldehyde (Sigma-Aldrich, #28908) in PBS, then transferred to 30% sucrose and frozen at −80 °C. Corpus callosum (CC), cortex, and hippocampus were dissected from the other hemisphere and flash-frozen in liquid nitrogen for biochemical analyses.

Immunohistochemistry and immunofluorescence microscopy

Frozen tissue was sectioned at 30 µm using a ThermoFisher Scientific Cyrotome FSE Cryostat and stored at 30 °C in cryoprotectant (25% glycerol, 25% ethylene glycol in PBS). Free-floating sections were incubated for 10 min at 70 °C in 10 mM sodium citrate, pH 6.0, 0.01%

Tween 20, followed by 3 washes with PBS containing 0.1% Tween 20 (PBST). Sections were then incubated in blocking solution (5% goat serum, 0.1% BSA, 0.1% Triton X-100 in PBS) for 2 h at RT, and overnight at 4 °C in primary antibodies (Table 1) diluted in blocking solution. One section was incubated in blocking buffer without primary antibody as a control for non-specific binding of secondary antibodies. Sections were then washed and incubated with AlexaFluor 488-, 546-, or 647-conjugated secondary antibodies (1:500 in blocking solution) for 2 h at RT in the dark. After 3 washes with PBST, sections were stained with 1 µg/mL diamidino-2-phenylindole dihydrochloride (DAPI) for 7 min, washed 2 times with PBST, and mounted on poly-L-Lysine coated slides (ProSciTec, #G312P-W) using ProLong Gold anti-fade (Life Technologies, #P36935). For NG2/Olig2 staining, permeabilization was performed using 0.3% Triton X-100 in PBS for 10 min, blocking solution was 5% BSA, 0.1% Triton X-100 in PBS, primary antibodies were diluted in PBS containing 2.5% BSA and 0.05% Triton X-100, and sections were incubated with donkey anti-goat Alexa-488 and goat anti-rabbit Alexa-546 antibodies sequentially for 2 h each time, with four washes in between. Slides were imaged using an Olympus Virtual Slide microscope VS1200, Zeiss Axio Scan.Z1, or Leica SP8 microscope. In all cases, the experimental operator and analyser remained blinded to the treatment groups until after quantification.

ASPA-positive cells were counted using the particle analysis function in ImageJ (v. 1.53b), based on size (7.50-100.00 μm^2) and circularity (0.25-1.00). NG2/Olig2 double-positive cells were counted manually by a blinded observer. Cell density was calculated as cell number divided by area. GFAP and Iba1 staining were quantified as the area of CC occupied by the fluorescent stain.

Table 1. Primary antibodies used for immunofluorescence (IF) and western blotting (WB).

Primary antibody	Supplier	Dilution	Cat. #	RRID
Rabbit anti-ASPA	Abcam	1:1000 (WB/IF)	ab223269	None found
Rabbit anti-CerS2	Thermo Fisher	1:1000 (WB)	PA5-55560	AB_2643295
Rabbit anti-MBP	Abcam	1:1000 (WB)	ab40390	AB_1141521
Rabbit anti-MOG	Abcam	1:1000 (WB)	ab32760	AB_2145529
Rabbit anti-PLP	Abcam	1:1000 (WB)	ab28486	AB_776593
Mouse anti-CNP [11-5B]	Abcam	1:1000 (WB)	ab6319	AB_2082593
Rabbit anti-β-actin	Abcam	1:5000 (WB)	ab8227	AB_2305186
Rabbit anti-NG2	Sigma Aldrich	1:500 (IF)	ZRB5320	None found
Goat anti-Olig2	R&D Systems	1:500 (IF)	AF2418	AB_2157554
Rabbit Anti-Iba1	Abcam	1:1000 (IF)	ab178847	AB_2832244
Mouse anti-GFAP (GA5)	Cell Signalling Technology	1:1000 (IF)	3670S	AB_561049

Luxol fast blue/cresyl violet staining

Sections were mounted on poly-L-lysine-coated microscope slides and dried at 37 °C for 3 h. Sections were defatted for 5 h in 1:1 alcohol/chloroform, soaked in 95% ethanol for 30 min, then incubated for 16 h at 60 °C in 1 mg/ml luxol fast blue (Sigma-Aldrich, #S3382) in 95% ethanol. Sections were rinsed with 80% ethanol followed by water until clear, differentiated in 0.05% Li_2CO_3 (Sigma-Aldrich, #255823) solution for 30 min, rinsed in 70% ethanol and water, then counterstained with cresyl violet (1 mg/mL cresyl violet and 0.05 mg/mL oxalic acid), and rinsed successively in water, 70% ethanol, 95% ethanol, 100% ethanol and xylene. DPX mountant (Sigma-Aldrich, #06522) was used for mounting, and slides were dried overnight.

Western blotting

Tissue samples (10-20 mg) were homogenized at 4 °C in 0.4 mL radio-immunoprecipitation assay buffer (10 mM pH 7.4 Tris, 100 mM NaCl, 100 mM sodium pyrophosphate, 0.5% sodium deoxycholate, 1% Triton-X, 10% glycerol, 1 mM EDTA, 1 mM NaF, 0.1% SDS) supplemented with Complete Mini, EDTA-free Protease Inhibitor Cocktail (Sigma, #11836170001) and

phosphatase inhibitors (5 mM β-glycerophosphate, 2 mM sodium orthovanadate, and 5 mM NaF), using a Biospec mini bead beater with acid-washed glass beads (425-600 μm). Extracts were cleared by centrifugation at 1000 g for 15 min, at 4 °C, and protein concentration was measured by bicinchoninic acid assay (ThermoFisher Scientific, #23225). Protein lysates were resolved on Bolt™ 4-12% Bis-Tris Plus gels (ThermoFisher Scientific) and transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), then washed three times with TBST and incubated overnight (4 °C) with primary antibody (Table 1) in TBST containing 3% BSA. Membranes were washed three times with TBST, incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signalling #7074, RRID: AB_2099233, or #7076, RRID: AB_330924) in blocking buffer for 1 h, and washed again before imaging. Signal was developed with ECL chemiluminescence reagent (EMD Millipore), and images captured on a Bio-Rad ChemiDoc Touch. When necessary, membranes were stripped using 15 g/L glycine, 0.1% SDS, 1% Tween 20, pH 2.2, then re-probed with anti-actin as a control for protein loading. Densitometry was performed using Bio-Rad Image Lab software Version 5.2. A common loading control sample was included on every gel to control for variation in relative band intensities on different blots.

Lipid quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Lipids were extracted from RIPA lysates of the CC (~100 μg protein, respectively) using a two-phase methyl-tert-butyl ether (MTBE):methanol:water (10:3:2.5 v/v/v) extraction procedure (Couttas et al., 2020; Matyash, Liebisch, Kurzchalia, Shevchenko, & Schwudke, 2008). Internal standards (2 nmole each of d18:1/17:0 ceramide, and d18:1/12:0 sulfatide, d18:1/12:0 HexCer, 1 nmole d18:1/12:0 SM, and 200 pmoles of d17:1 sphingosine and d17:1 S1P) were added at the start of the extraction procedure. Lipids were detected by multiple

reaction monitoring on a TSQ Altis mass spectrometer with Vanquish HPLC (ThermoFisher Scientific), operating in positive ion mode. The $[M + H]^+$ mass-to-charge (*m/z*) ratio was used for all precursor ions. Product ions were *m/z* 184.0 *m/z* for SM, 250.2 for d17:1 sphingosine and d17:1 S1P, and 264.3 for all other sphingolipids. Lipids were resolved on a 3 × 150 mm XDB-C8 column (5 µM particle size) (Agilent) at flow rate 0.4 mL/min. Mobile phase A was 0.2% formic acid, 2 mM ammonium formate in water; and B was 0.2% formic acid, 2 mM ammonium formate in methanol. Total run time was 24 min, starting at 80% B and holding for 2 min, increasing to 100% B from 2 - 14 min, holding at 100% until 20.5 min, then returning to 80% B at 21 min, and holding at 80% B for a further 3 min. Peaks were integrated using TraceFinder 4.1 software (ThermoFisher) and expressed as ratios to the relevant internal standard. Lipid levels were determined with reference to standard curves created by extracting spiked samples, comprising one or two lipids of each class at five different concentrations, together with the internal standards, in 1% (w/v) fatty acid free BSA.

Scanning Electron Microscopy

Male SphK2$^{-/-}$ mice or their WT littermates, at 2 or 15 months of age, were perfused trans-cardially with sterile PBS, then fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.15 M PBS). Brains were removed and post-fixed overnight with the same fixative, then stored at 4 ºC in 30% sucrose cyroprotectant. The brain was serially sectioned into 1 mm thick coronal sections. Blocks (1 mm x 2 mm) were dissected from the coronal section located 7 mm posterior to the olfactory bulbs. Hippocampal blocks were dissected from the Cornu Ammonis 1 subregion. CC and cortical blocks were dissected from the region superior to the hippocampus, corresponding to the somatosensory cortex of the parietal lobe. Blocks (1 mm x 2 mm) were immersion fixed for 1 h in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, washed in 0.1 M phosphate buffer, then subjected to secondary fixation in

1% osmium tetroxide in 0.1 M phosphate buffer, followed by dehydration through graded alcohols, and Epon (medium) resin infiltration. Following polymerization for 48 h at 60 °C, blocks were sectioned at 90 nm thickness on an ultramicrotome (Ultracut 7, Leica), mounted on copper grids, and post-stained in 2% uranyl acetate and Reynold's lead citrate. Images were captured using a backscatter detector on a scanning electron microscope (Zeiss Sigma HD FEG SEM). G-ratios were calculated as the axon lumen diameter divided by the outer diameter (axon lumen plus myelin sheath). These measurements were taken from 30-60 axons per region per mouse, with 3 mice per age/genotype, using ImageJ software. Measurements were taken perpendicular to the longest axis of axons in cross section, to compensate for axons cross-sectioned in an oblique plane. The operator was blinded to sample groups.

Statistical Analysis

Results were analysed using two-way ANOVA (type III) with genotype (WT or SphK2$^{-/-}$) and treatment (ctrl, 6W, 6+2W) as the independent variables, followed by Bonferroni's post-hoc tests for the effect of different treatments within each genotype (comparing Ctrl to 6W and 6W to 6+2W), or the effect of genotype under each treatment condition. For the 4 week remyelination experiment using only SphK2$^{-/-}$ mice, one-way ANOVA with Dunnett's post-test was used. For mean g-ratio measurements, two-way ANOVA was used with genotype and age as the independent variables. Least squares regression was used to create lines of best fit for g-ratio against axon diameter (no weighting), and the sum-of-squares F test was applied to determine if the fit of the lines differed significantly between WT and SphK2$^{-/-}$ mice. These analyses were all performed using GraphPad PRISM. The *car* and *multcomp* packages in R were used to run two-way ANOVA on each lipid shown in Table 2, and *p* values were adjusted for multiple comparisons with the Benjamini-Hochberg false discovery rate correction.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

SphK2 is essential for remyelination following cuprizone withdrawal

To determine if SphK2 is required for protection of oligodendrocytes and myelin, and for spontaneous remyelination following cuprizone withdrawal, SphK2$^{-/-}$ mice and their WT littermates were fed cuprizone for 6 weeks and culled immediately (6W), or returned to a normal chow diet (i.e. removal of cuprizone from the diet) for 2 weeks prior to culling (6+2W). These were compared to mice fed a normal chow diet for 6 weeks (Ctrl). Luxol fast blue staining showed robust demyelination in the corpus callosum (CC) of cuprizone-fed WT mice, and remyelination was apparent following cuprizone withdrawal (Figure 1a). Demyelination appeared more pronounced in SphK2$^{-/-}$ mice, and remyelination was less apparent following cuprizone withdrawal.

To gain a more quantitative assessment of myelin content, we performed western blotting for the myelin markers myelin basic protein (MBP), myelin proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) in CC (Figure 1b-d) and cortex (Figure 2a-c) homogenates. Myelin protein levels did not differ between untreated WT and SphK2$^{-/-}$ mice in the CC, although PLP and MOG levels were significantly lower in the cortex of untreated SphK2$^{-/-}$ mice. Cuprizone feeding produced a marked loss of all four myelin markers in both CC and cortex, and a significant effect of both genotype and treatment was observed for all four markers in two-way ANOVA (Figure 1d and 2c). Following cuprizone withdrawal, MBP, CNP and PLP levels increased significantly in the CC of WT mice ($p < 0.001$, $p = 0.003$, and $p = 0.04$, respectively, 6W compared to 6+2W), whereas none of these markers increased in SphK2$^{-/-}$ mice after cuprizone withdrawal. Myelin marker levels did not increase significantly in the cortex of WT or SphK2$^{-/-}$ mice after cuprizone withdrawal, indicating that remyelination proceeds more rapidly in the CC than the

cortex. Nonetheless, MBP, CNP, and PLP levels were significantly lower in cortex of SphK2$^{-/-}$ compared to WT mice in the 6+2W group, indicative of a more severe myelin deficit in SphK2$^{-/-}$ mice.

SphK2 protects against loss of mature oligodendrocytes

We next determined if cuprizone administration differentially affected oligodendrocyte number in WT versus SphK2$^{-/-}$ mice, by counting cells positive for the mature oligodendrocyte marker aspartoacylase (Aspa) (Madhavarao et al., 2004; Y. Zhang et al., 2014) (Figure 3a). Cuprizone feeding produced a pronounced depletion of Aspa-positive cells in CC (Figure 3b) and cortex (Figure 3c), which was more pronounced in SphK2$^{-/-}$ compared to WT mice. Aspa-positive cell density was reduced 61% in CC of WT mice, and 84% in SphK2$^{-/-}$ mice (2-way ANOVA, effect of genotype: $F = 8.2$, $p = 0.008$; treatment: $F = 87.7$, $p < 0.001$; interaction: $F = 1.7$, $p = 0.19$). In the cortex, Aspa-positive cell density declined 50% in WT and 72% in SphK2$^{-/-}$ mice after 6 weeks of cuprizone administration (2-way ANOVA, effect of genotype: $F = 26.7$, $p < 0.001$; treatment: $F = 56.1$, $p < 0.001$; interaction: $F = 1.5$, $p = 0.25$).

Two weeks after cuprizone withdrawal, mature oligodendrocyte density in the CC had returned to 80% of the control condition in WT mice, and 77% of the control in SphK2$^{-/-}$ mice ($p < 0.001$ for both genotypes comparing 6W to 6+2W) (Figure 3b). In contrast, oligodendrocyte density in the cortex did not recover after 2 weeks of cuprizone withdrawal and remained significantly lower in SphK2$^{-/-}$ compared to WT mice (Figure 3c). Oligodendrocyte density did not differ between untreated WT and SphK2$^{-/-}$ mice in CC or cortex.

Cuprizone administration is associated with an expansion of oligodendrocyte progenitor cells (OPCs), as a response to loss of mature oligodendrocytes (Gudi et al., 2009; Mason et al.,

2000). OPCs were quantified as cells positive for both the pan-oligodendrocyte lineage transcription factor Olig2 and the OPC marker NG2 proteoglycan (Zhan et al., 2020) (Figure 3d). $NG2^{+}/Olig2^{+}$ cell density doubled in cuprizone-fed mice and remained elevated 2 weeks after cuprizone withdrawal (Figure 3e). This response was not notably affected by SphK2 deficiency (2-way ANOVA, genotype: $F = 2.2$, $p = 0.15$; treatment: $F = 26.4$, $p < 0.001$; interaction: $F = 0.93$, $p = 0.41$).

In agreement with Aspa-positive cell counts, Aspa protein levels in the CC were decreased in the 6W group and partially restored in the 6+2W group, for both WT and $SphK2^{-/-}$ mice ($p < 0.001$ for both WT and $SphK2^{-/-}$ mice, 6W vs 6+2W) (Figure 1b-c). Another mature oligodendrocyte marker, ceramide synthase 2 (CerS2) (Couttas et al., 2016; Kremser et al., 2013), which is required for myelin lipid synthesis, showed a similar pattern of reduced levels in the 6W group, increasing substantially in the 6+2W group. In contrast to the myelin markers, a significant effect of genotype was not observed for Aspa and CerS2 in two-way ANOVA (Figure 1d), supporting the hypothesis that production of new mature oligodendrocytes from OPCs is unaffected in $SphK2^{-/-}$ mice, despite the lack of remyelination.

No remyelination in the CC of $SphK2^{-/-}$ mice four weeks after cuprizone withdrawal

Since mature oligodendrocyte numbers recovered in the CC of SphK2-/- mice after cuprizone withdrawal, we tested if remyelination in the CC of $SphK2^{-/-}$ mice is simply delayed relative to WT mice. $SphK2^{-/-}$ mice were fed chow containing cuprizone for 6 weeks, then allowed 4 weeks for recovery on normal chow before assessing myelin content (6+4W). Western blotting showed a significant decrease of MBP, CNP, MOG and PLP in the CC with cuprizone administration (6W group). However, these markers did not recover 4 weeks after cuprizone withdrawal, whereas levels of the mature oligodendrocyte markers Aspa and CerS2 rebounded

significantly (Figure 4a-b). Luxol fast blue staining further verified the lack of remyelination in SphK2$^{-/-}$ mice at 4 weeks after cuprizone withdrawal (Figure 4c). These results suggest that SphK2$^{-/-}$ is essential for remyelination, rather than simply delaying remyelination following cuprizone withdrawal.

Astrogliosis does not resolve after cuprizone withdrawal in SphK2$^{-/-}$ mice

Astrogliosis and microgliosis in the CC, visualised with the markers GFAP and Iba1, respectively, are well-established responses to cuprizone feeding (S. Kim et al., 2018; Zhan et al., 2020). A pronounced increase in GFAP (Figure 5a-b) and Iba1 (Figure 5c-d) staining was observed in the CC of cuprizone-fed mice. GFAP staining subsided following cuprizone withdrawal in WT (p = 0.0025, 6W vs 6+2W, Bonferroni's post-test) but not SphK2$^{-/-}$ (p > 0.99) mice, indicating deficient resolution of astrogliosis in SphK2$^{-/-}$ mice. Iba1 staining declined significantly in both WT and SphK2$^{-/-}$ mice after cuprizone withdrawal (both genotypes p < 0.001, 6W vs 6+2W).

Sphingosine and ceramide levels remain elevated in SphK2$^{-/-}$ mice after cuprizone withdrawal

To determine how the absence of SphK2 affects sphingolipid metabolism during and after cuprizone withdrawal, we quantified levels of S1P, its precursors sphingosine and ceramide, and essential myelin sphingolipids derived from ceramide: sulfatide, hexosylceramide (HexCer), and sphingomyelin (SM) (Figure 6a). Mean S1P levels did not change significantly with cuprizone treatment in WT mice, declining 29% in CC of WT mice with cuprizone treatment (not significant), and increasing 60% following cuprizone withdrawal (not significant) (Figure 6b). Compared to WT mice, S1P levels were an order of magnitude lower in CC of untreated SphK2$^{-/-}$ mice. Remarkably, however, S1P levels were 10-fold higher in the 6W SphK2$^{-/-}$ compared to the Ctrl SphK2$^{-/-}$ group (p < 0.001), and remained elevated in the

6+2W group. Sphingosine was unchanged in WT mice across all treatment groups, but increased 3.2-fold in SphK2$^{-/-}$ mice with cuprizone treatment (p = 0.0014, Ctrl vs 6W). Compared to WT mice, sphingosine content was 4-fold higher in SphK2$^{-/-}$ mice in the 6W treatment group (p = 0.0003), and 3-fold higher in the 6+2W group (p = 0.025) (Figure 6c). Mean ceramide levels increased 50% in WT mice with 6W cuprizone feeding (not significant), then declined 2.5-fold in the 6+2W group (p = 0.0008 compared to 6W) (Figure 6d). Compared to WT mice, total ceramide levels were not significantly higher in untreated or 6W cuprizone-treated SphK2$^{-/-}$ mice. However, ceramide levels did not decrease in SphK2$^{-/-}$ mice following cuprizone withdrawal, resulting in 2.3-fold higher levels in CC of SphK2$^{-/-}$, relative to WT mice, in the 6+2W group (p = 0.003). Sphingosine and ceramide levels were significantly affected by both genotype and treatment in 2-way ANOVA (Figure 6h).

SM and HexCer levels were not significantly affected by treatment or genotype (Figure 6e-f), whereas the myelin lipid sulfatide decreased with cuprizone feeding in both WT (30% decrease, p = 0.021, 6W compared to Ctrl) and SphK2$^{-/-}$ mice (45% decrease, p = 0.003) (Figure 6g). An overall effect of genotype on sulfatide levels was observed (F = 11.4, p = 0.012), as levels were lower in SphK2$^{-/-}$ mice. However this difference was not significant in individual treatment group comparisons. Results for individual lipid species are shown in Table 2.

Myelin is thinner in aged SphK2$^{-/-}$ mice

Since SphK2$^{-/-}$ mice did not remyelinate following cuprizone withdrawal, we hypothesized that these mice may show age-dependent myelin loss, due to impaired physiological myelin turnover. Mature oligodendrocyte density in the cortex and CC did not differ between WT and SphK2$^{-/-}$ mice at 20 months of age (Figure 7a-b), and western blotting did not reveal any significant difference in levels of major myelin proteins in the cortex (Figure 7c-d). However,

analysis of scanning electron microscopy images revealed a significant reduction in myelin thickness (increased g-ratio) across all axon diameters in the cortex and CC of 15 month old SphK2$^{-/-}$ mice (Figure 7e,f). Myelin thickness did not differ between WT and SphK2$^{-/-}$ mice at 2 months of age (Figure 7g,h). The line of best fit for g-ratio against axon diameter differed significantly between WT and SphK2$^{-/-}$ mice at 15 months in both cortex and CC (both $p < 0.001$), but did not differ significantly at 2 months (both $p > 0.05$). Mean g-ratio in the cortex and CC was significantly higher at 15 months in SphK2$^{-/-}$ mice (Figure 7i-j).

Discussion

Despite considerable interest in the putative oligodendrocyte-protective and pro-myelinating properties of Fingolimod (Alme et al., 2015; Bechet, O'Sullivan, Yssel, Fagan, & Dev, 2020; H. J. Kim et al., 2011; S. Kim et al., 2018; Nystad et al., 2020; J. Zhang et al., 2015), the role for endogenous S1P and sphingosine kinases in myelination has not yet been elucidated. SphK2 is the dominant isoform catalysing S1P synthesis in the CNS (Lei et al., 2017). This study establishes that endogenous SphK2 protects against cuprizone-mediated loss of mature oligodendrocytes and is essential for remyelination following cuprizone intoxication. Myelin protein levels and oligodendrocyte number are normal in the CC of treatment-naïve SphK2$^{-/-}$ mice at 5 months of age, however loss of mature oligodendrocytes was more severe in SphK2$^{-/-}$ compared to WT mice after 6 weeks of cuprizone feeding. Remyelination was observed in the CC of WT mice at two weeks after cuprizone withdrawal, whereas no remyelination was observed in the CC of SphK2$^{-/-}$ mice even after a 4-week recovery period. The well-established accumulation of NG2-positive OPCs in cuprizone-treated mice (Gudi et al., 2009; Mason et al., 2000) was unaffected by SphK2 deletion, and mature oligodendrocyte numbers recovered robustly in the CC of both WT and SphK2$^{-/-}$ mice following cuprizone withdrawal, indicating that SphK2 is not necessary for expansion and maturation of OPCs *in vivo* but is necessary for the synthesis of new myelin by mature oligodendrocytes.

Oligodendrocyte apoptosis is reported to commence in the first or second week of cuprizone feeding, leading to notable myelin loss by 4 weeks (Doan et al., 2013; Gudi et al., 2009; Mason et al., 2000). Greater loss of mature oligodendrocytes in SphK2$^{-/-}$ mice with cuprizone feeding is very likely the result of increased apoptosis, although this was not directly measured. An important role for SphK2 in oligodendrocyte survival is supported by our recent work demonstrating that loss of SphK2 enhances age-dependent loss of oligodendrocytes in an

Alzheimer's disease mouse model (Lei et al., 2019). S1P is a pro-survival signalling molecule (Grassi et al., 2019; Hannun & Obeid, 2018) and SphK2 generally promotes cell proliferation and survival (Gao & Smith, 2011; Neubauer et al., 2016). This is not true of all cell types and scenarios, for example loss of SphK2 actually protects renal mesangial cells against stress-induced apoptosis (Hofmann, Ren, Schwalm, Pfeilschifter, & Huwiler, 2008). However, SphK2 overexpression in neurons confers resistance to stress through activation of autophagy (Song et al., 2017), whilst loss of SphK2 sensitizes to ischaemic brain injury (Pfeilschifter et al., 2011). It remains to be determined if endogenous SphK2 protects stressed oligodendrocytes *in vitro*.

S1P produced by SphK2 mediates its effects through autocrine or paracrine stimulus of S1P receptors (Choi & Chun, 2013; Grassi et al., 2019), and through intracellular binding targets such as histone deacetylases and peroxisome proliferator-activated receptor γ (PPARγ) (Hait et al., 2009; Parham et al., 2015). Fingolimod-phosphate and S1PR1-selective agonist CYM-5442 protect oligodendrocytes against apoptosis induced with cuprizone *in vivo* (S. Kim et al., 2018), implying a role for S1PR1 on CNS cells in oligodendrocyte survival. This protection probably results from agonism rather than functional antagonism of S1PR1, since cuprizone-mediated demyelination is significantly worse in mice lacking S1PR1 in oligodendrocytes (H. J. Kim et al., 2011). S1PR5 is very highly expressed by mature oligodendrocytes and mediates pro-survival S1P signalling *in vitro* (Jaillard et al., 2005). No publications have reported on the requirement for S1PR5 in oligodendrocyte protection or remyelination following cuprizone feeding, however S1PR5 deletion impeded remyelination mediated by the S1PR1/5 agonist Siponimod in a demyelination model using *Xenopus* oocytes (Mannioui et al., 2017). Studies currently underway in our laboratory will determine whether pharmacological S1P receptor

stimulus rescues oligodendrocyte survival during cuprizone feeding in SphK2$^{-/-}$ mice, and/or restores remyelination following cuprizone withdrawal.

Counting of OPCs and mature oligodendrocytes indicated that OPC expansion and maturation are not affected by SphK2 deficiency. Western blotting showed up-regulation of the oligodendrocyte cell markers ASPA and CerS2 in both WT and SphK2$^{-/-}$ mice following cuprizone withdrawal. In contrast, the myelin markers MBP, PLP, and CNP increased in WT mice 2 weeks after cuprizone withdrawal but did not recover in SphK2$^{-/-}$ mice even after 4 weeks of cuprizone withdrawal. These results suggest that SphK2 is essential for synthesis of new myelin rather than oligodendrocyte maturation. In accord with this, a recent study using an *ex vivo* nerve injury model demonstrated that S1P and PPARγ are down-regulated during conversion of Schwann cells to a repair phenotype, and up-regulated during re-conversion to a myelinating phenotype (Meyer Zu Reckendorf et al., 2020). Up-regulation of both was necessary for remyelination following nerve injury. S1P stimulates PPARγ-dependent lipogenic gene transcription (Meyer Zu Reckendorf et al., 2020; Parham et al., 2015), which is necessary for myelin synthesis. A limitation of our study is that we did not resolve whether the SphK2 that is required for remyelination is expressed by oligodendrocytes or another cell type. SphK2 deficiency in neurons or other glial cells could produce a lack of S1P receptor stimulus on oligodendrocytes. The related lipid lysophosphatidic acid, produced by dorsal root ganglion neurons, activates Schwann cell LPAR1 receptors to promote their migration (Anliker et al., 2013).

We previously demonstrated that S1P levels are ~15% of WT levels in the hippocampus and cortex of SphK2$^{-/-}$ mice (Lei et al., 2017; Lei et al., 2019). In this study, we report an equivalent reduction in S1P in the CC of treatment-naïve SphK2$^{-/-}$ mice. Surprisingly, S1P levels were an

order of magnitude higher in CC of cuprizone-treated SphK2$^{-/-}$ mice and remained elevated following cuprizone withdrawal. In contrast, S1P levels were modestly reduced in CC of cuprizone-fed WT mice and increased 60% after cuprizone withdrawal (not significant). Based on this result, greater loss of mature oligodendrocytes and deficient remyelination in SphK2$^{-/-}$ mice with cuprizone feeding cannot be attributed to bulk S1P deficiency. However, the loss of SphK2-dependent S1P synthesis might still impede oligodendrocyte functions through a deficiency in autocrine, localised S1P receptor stimulus. Alternatively, SphK2 may be essential for intracellular signalling pathways regulating myelination. For example, S1P produced by nuclear-localised SphK2 directly inhibits type I histone deacetylases in neurons, leading to up-regulation of neurotrophic genes such as brain derived neurotrophic factor, and enhanced hippocampal plasticity (Hait et al., 2014). These pathways cannot be rescued by SphK1, which has no nuclear import signal (Igarashi et al., 2003). It is possible, although unverified, that increased S1P in SphK2$^{-/-}$ mice fed cuprizone is attributed to high SphK1 activity in reactive astrocytes and microglia (Fischer et al., 2011; Nayak et al., 2010), which may not trigger the necessary S1P-dependent responses in oligodendrocytes. We were unable to quantify SphK1 protein levels in this study, since all SphK1 antibodies that we tested failed to show loss of the putative SphK1 band in SphK1$^{-/-}$ mouse brain tissue.

Pronounced accumulation of the SphK2 substrate sphingosine was observed in cuprizone-fed SphK2$^{-/-}$ mice. Sphingosine remained elevated following cuprizone withdrawal in SphK2$^{-/-}$ mice, as did its precursor ceramide. It is likely that ceramide and sphingosine accumulation in cuprizone-fed SphK2$^{-/-}$ mice results from impaired degradation of myelin sphingolipids. GalCer, sulfatide, and SM are catabolised in lysosomes to sphingosine which, due to its biophysical properties, can escape lysosomes and act as a substrate for new ceramide synthesis

in the ER. Complete sphingolipid degradation requires conversion of sphingosine to S1P by SphK1 or SphK2, then irreversible cleavage of S1P by S1P lyase (Hannun & Obeid, 2018).

In direct contrast to S1P, sphingosine is a pro-apoptotic signalling molecule (Kagedal, Zhao, Svensson, & Brunk, 2001; Woodcock et al., 2010) that activates specific protein kinase C isoforms and inhibits pro-survival signalling by promoting phosphorylation of the abundant scaffolding protein 14-3-3 (Hamaguchi et al., 2003; Woodcock et al., 2010). Ceramide is also a pro-apoptotic lipid that promotes cell death in oligodendrocytes (S. Kim, Steelman, Zhang, Kinney, & Li, 2012; Plo et al., 1999). Previous reports have demonstrated significantly increased sphingosine and ceramide in the cuprizone (S. Kim et al., 2012; Yoo et al., 2020) and experimental autoimmune encephalitis models (Miller et al., 2017). A recent study demonstrated that ceramide resulting from SM hydrolysis impedes remyelination after cuprizone withdrawal (Yoo et al., 2020). The authors proposed that excess ceramides cause oligodendrocyte death and myelin decompaction. Sphingosine and ceramide accumulation might therefore contribute significantly to enhanced loss of mature oligodendrocytes with cuprizone feeding and failure of remyelination in SphK2$^{-/-}$ mice. Alternatively, or in parallel, SphK2 deficiency in cells phagocytosing myelin could block remyelination through impaired catabolism of myelin lipids, as observed in mice lacking the microglial lipoprotein receptor Trem2 (Cantoni et al., 2015; Cignarella et al., 2020).

Our observation of significantly reduced sulfatide, but not HexCer or SM levels, with cuprizone feeding is in agreement with a recent publication (Yoo et al., 2020). Decreased sulfatide is almost certainly a direct consequence of myelin loss. Unlike HexCer and SM, sulfatide is unique to myelin in the nervous system. HexCer comprises both the myelin-specific

lipid GalCer and the ubiquitous glucosylceramide, which are positional isomers and indistinguishable in reverse phase LC-MS/MS analyses.

We hypothesized that the remyelination deficit in SphK2$^{-/-}$ mice would affect myelin content and integrity with ageing, as physiological myelin replacement over time is impaired. There was no difference in myelin protein levels in 20 month old mice, but SphK2$^{-/-}$ mice exhibited substantially increased g-ratios, indicative of thinner myelin, at 15 months of age. Aspa-positive cell density was equivalent between WT and SphK2$^{-/-}$ mice, indicating that SphK2 deficiency affects myelin maintenance rather than oligodendrocyte survival with ageing. A very modest but statistically significant reduction in myelin thickness was reported in the CC of 3 week old mice lacking S1PR1 in mature oligodendrocytes (Dukala & Soliven, 2016). It would be interesting to determine if this phenotype is more pronounced with ageing, since these mice also displayed increased demyelination with cuprizone (H. J. Kim et al., 2011). Myelin thickness in SphK2$^{-/-}$ mice was equivalent to WT mice at 2 months of age, implying that developmental myelination is not notably affected by SphK2 deficiency. We speculate that S1P production and lipid flux through SphK1, which is highly expressed during development (Liu et al., 2000), is sufficient for developmental myelination. Alternatively, the requirement for SphK2 in remyelination following cuprizone intoxication may reflect distinct functional roles for SphK1 and SphK2. Unlike developmental myelination, remyelination after a demyelinating insult requires clearance of myelin debris by phagocytic cells (Cantoni et al., 2015). A defect in myelin clearance resulting from SphK2 deficiency could affect myelin turnover with ageing.

Conclusions

Overall, these results identified essential roles for SphK2 in protecting against loss of mature oligodendrocytes during cuprizone feeding, and spontaneous remyelination following cuprizone withdrawal. Future work will clarify whether SphK2 promotes remyelination through production of S1P that stimulates S1P receptors on oligodendrocytes, or whether the requirement for SphK2 in sphingolipid catabolism is necessary to clear myelin debris and thereby facilitate remyelination. This research is important for establishing whether S1P receptor signalling is essential for remyelination following injury, and therefore whether agonists of S1PRs, already used in MS therapy, possess remyelinating properties.

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Figure Legends

Figure 1. SphK2 deletion impairs remyelination following cuprizone withdrawal.

(a) Luxol fast blue (light blue)/cresyl violet (purple) staining of sagittal sections from WT or SphK2$^{-/-}$ mice fed control chow (Ctrl) or cuprizone for 6 weeks (6W), or cuprizone for 6 weeks then normal chow for 2 weeks (6+2W). Images on left show the body and splenium of the CC above the hippocampus (HIP). Images on right show an enlarged view of the splenium, where demyelination is clearly apparent (marked by arrows). Scale bar, 200 μm. (b) Western blots, (c) densitometric quantification, and (d) results of two-way ANOVA for myelin and oligodendrocyte markers in the CC. Protein levels were normalised to β-actin in each sample, n = 6 mice/group. Box and whisker plots show full data range with 25th – 75th percentile boxed, and horizontal bar marking the median. Ctrl, open boxes; 6W, hatched boxes; 6+2W, grey boxes. Statistical significance was determined by two-way ANOVA with results of Bonferroni's post-tests shown on the graphs: WT vs SphK2$^{-/-}$ mice (*$p < 0.05$, ** $p < 0.01$; *** $p < 0.001$); Ctrl vs 6W and 6W vs 6+2W (# $p < 0.05$, ## $p < 0.01$; ### $p < 0.001$).

Figure 2. SphK2 protects against myelin loss in the cortex.

(a) Western blots, (b) densitometric quantification, and (c) results of two-way ANOVA for myelin and oligodendrocyte markers in the cortex of WT or SphK2$^{-/-}$ mice fed control chow (Ctrl, white boxes) or cuprizone for 6 weeks (6W, hatched boxes), or cuprizone for 6 weeks then normal chow for 2 weeks (6+2W, grey boxes). Protein levels were normalised to β-actin in each sample, n = 5 - 6 mice/group. Box and whisker plots show full data range with 25th – 75th percentile boxed, and horizontal bar marking the median. Results of Bonferroni's post-tests are shown on the graphs: WT vs SphK2$^{-/-}$ mice (*$p < 0.05$, ** $p < 0.01$; *** $p < 0.001$); Ctrl vs 6W and 6W vs 6+2W (# $p < 0.05$, ## $p < 0.01$; ### $p < 0.001$).

Figure 3. Loss of SphK2 sensitizes to oligodendrocyte loss induced by cuprizone.

(a) Representative immunofluorescence image for ASPA (green) and DAPI (blue). Scale bar, 300 µm. (b,c) Quantified ASPA-positive cell density (cells per mm^2) in the CC (b) and cortex (c) of WT or $SphK2^{-/-}$ mice (6 mice/group) fed control chow (Ctrl) or cuprizone for 6 weeks (6W), or cuprizone for 6 weeks then normal chow for 2 weeks (6+2W). (d) Representative immunofluorescence image for Olig2 (green) and NG2 (red). Arrowheads indicate NG2-positive OPCs. Scale bar, 100 µm. (e) Quantification of NG2/Olig2-positive cell density in the CC. Box and whisker plots show full data range with 25th – 75th percentile boxed, and horizontal bar marking the median (6 mice/group). Statistical significance was determined by two-way ANOVA with results of Bonferroni's post-tests shown on the graphs: WT vs $SphK2^{-/-}$ mice (*$p < 0.05$, ** $p < 0.01$; *** $p < 0.001$); Ctrl vs 6W and 6W vs 6+2W (# $p < 0.05$, ## $p < 0.01$; ### $p < 0.001$).

Figure 4. No remyelination 4 weeks after cuprizone withdrawal in $SphK2^{-/-}$ mice.

(a) Western blots and (b) densitometric quantification of myelin and oligodendrocyte markers in the CC of $SphK2^{-/-}$ mice fed normal chow (Ctrl), cuprizone for 6 weeks (6W), or cuprizone for 6 weeks then normal chow for 4 weeks (6+4W). Protein levels were normalised to β-actin in each sample. Graphs show mean ± SEM (n = 5). Statistical significance was determined by one-way ANOVA with Dunnett's post-test (## $p < 0.01$; ### $p < 0.001$). (c) Representative images for luxol fast blue/cresyl violet staining of the CC. HIP: hippocampus. Scale bar, 200 µm.

Figure 5. Sustained astrogliosis in $SphK2^{-/-}$ mice following cuprizone withdrawal. Representative images (a,c) and quantified area of staining (b,d) for GFAP (a,b) and Iba1 (c,d) in the CC of WT or $SphK2^{-/-}$ mice fed control chow (Ctrl) or cuprizone for 6 weeks (6W), or

cuprizone for 6 weeks then normal chow for 2 weeks (6+2W). Scale bar, 50 μm. Box and whisker plots show full data range with 25th – 75th percentile boxed, and horizontal bar marking the median (6 mice/group). Main effect two-way ANOVA results are shown below each graph, and results of Bonferroni's post-tests are shown on the graphs: Ctrl vs 6W and 6W vs 6+2W (# $p < 0.05$, ## $p < 0.01$; ### $p < 0.001$). All comparisons between WT and SphK2$^{-/-}$ mice were non-significant.

Figure 6. High levels of sphingosine and ceramide in SphK2$^{-/-}$ mice following cuprizone withdrawal.

(a) Basic sphingolipid pathway in oligodendrocytes. SM, sphingomyelin; HexCer, hexosylceramide. HexCer is comprised of both glucosylceramide and galactosylceramide (GalCer). GalCer is an abundant and essential constituent of myelin. (b-g) Levels of (b) S1P, (c) sphingosine, (d) ceramide, (e) SM, (f) HexCer, and (g) sulfatide in CC of WT or SphK2$^{-/-}$ mice fed control chow (Ctrl) or cuprizone (6W) for 6 weeks, or cuprizone for 6 weeks then normal chow for 2 weeks (6+2W). Box and whisker plots show full data range with 25th – 75th percentile boxed, and horizontal bar marking the median, n = 6 - 8 mice/group. (h) Results of two-way ANOVA for each lipid class. Results of Bonferroni's post-tests are shown on the graphs: WT vs SphK2$^{-/-}$ mice (*$p < 0.05$, ** $p < 0.01$; *** $p < 0.001$); Ctrl vs 6W and 6W vs 6+2W (# $p < 0.05$, ## $p < 0.01$; ### $p < 0.001$).

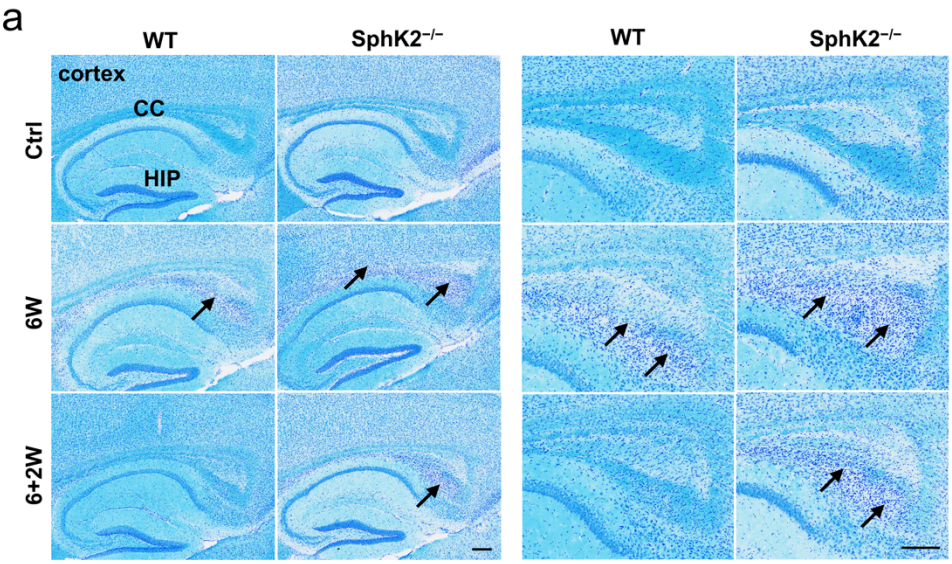
Figure 7. Thinner myelin in aged SphK2$^{-/-}$ mice.

(a-b) Density of Aspa-positive cells in the cortex (a) and CC (b) of 20 month old WT or SphK2$^{-/-}$ mice (6-7 mice/group). (c) Western blots and (d) densitometric quantification of myelin markers in cortical tissue from 20 month old mice (6 mice/group). Unpaired t-tests were used

to assess statistical significance of comparisons (ns = not significant). (e-h) G-ratio against axon diameter for myelinated axons in the cortex (e,g) and CC (f,h) of 15 month old (e,f) or 2 month old (g,h) mice (measurements from 3 mice per group). Lines of best fit are shown (WT, solid line and open circles; SphK2$^{-/-}$ dashed line and closed squares). (i-j) Mean g-ratio in cortex (i) and CC (j) of 3 mice per group. Results were analysed by two-way ANOVA with Bonferroni's post-test (***$p < 0.001$). ANOVA results are shown below the graphs. (k) Example EM image showing myelinated axons in a 15 month old WT mouse. Scale bar, 2 μM.

Figure 1

a



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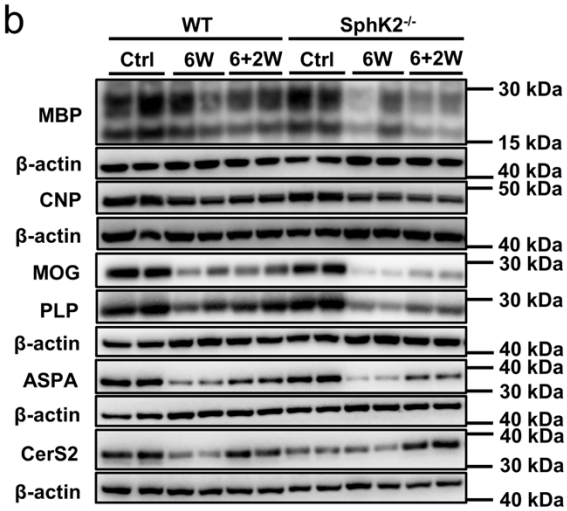

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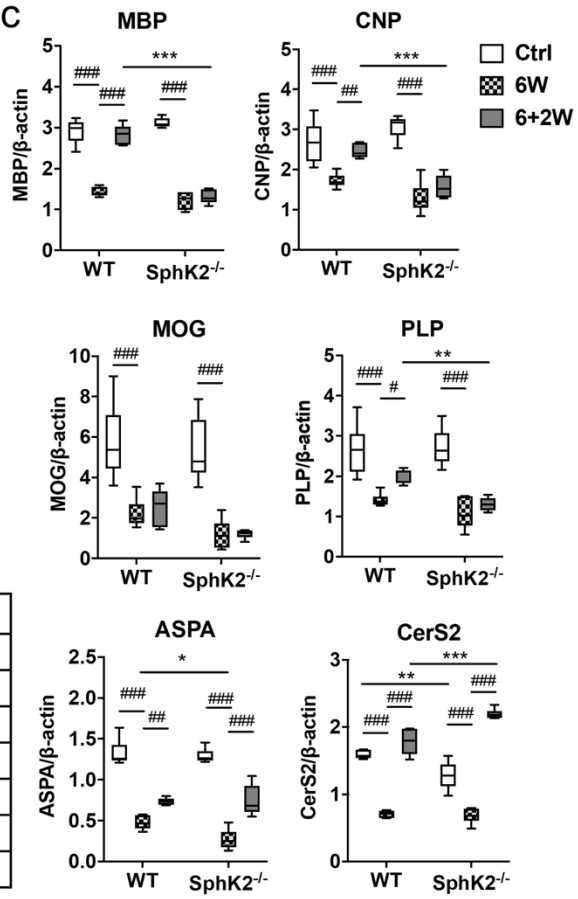
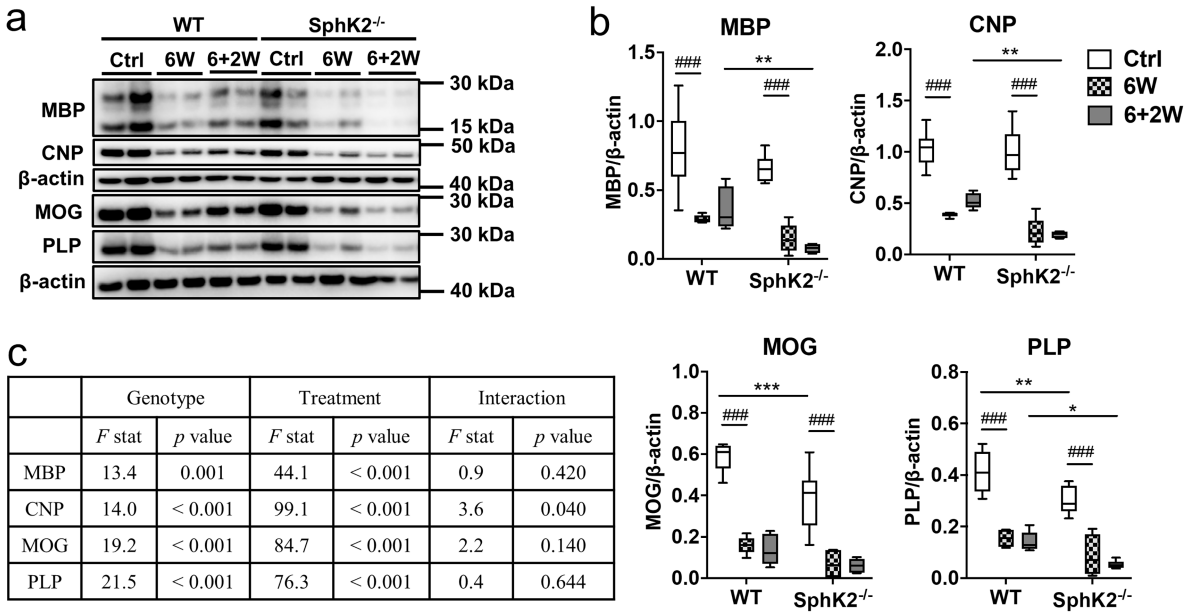


d

	Genotype		Treatment		Interaction	
	F stat	*p* value	*F* stat	*p* value	*F* stat	*p* value
MBP	58.0	< 0.001	214.2	< 0.001	61.3	< 0.001
CNP	8.1	0.008	53.5	< 0.001	11.7	< 0.001
MOG	5.9	0.022	42.9	< 0.001	0.5	0.610
PLP	7.2	0.012	47.3	< 0.001	3.3	0.052
ASPA	3.5	0.070	173.5	< 0.001	2.4	0.111
CerS2	0.3	0.579	273.8	< 0.001	21.5	< 0.001

Figure 2



c

	Genotype		Treatment		Interaction	
	F stat	*p* value	*F* stat	*p* value	*F* stat	*p* value
MBP	13.4	0.001	44.1	< 0.001	0.9	0.420
CNP	14.0	< 0.001	99.1	< 0.001	3.6	0.040
MOG	19.2	< 0.001	84.7	< 0.001	2.2	0.140
PLP	21.5	< 0.001	76.3	< 0.001	0.4	0.644

Figure 3

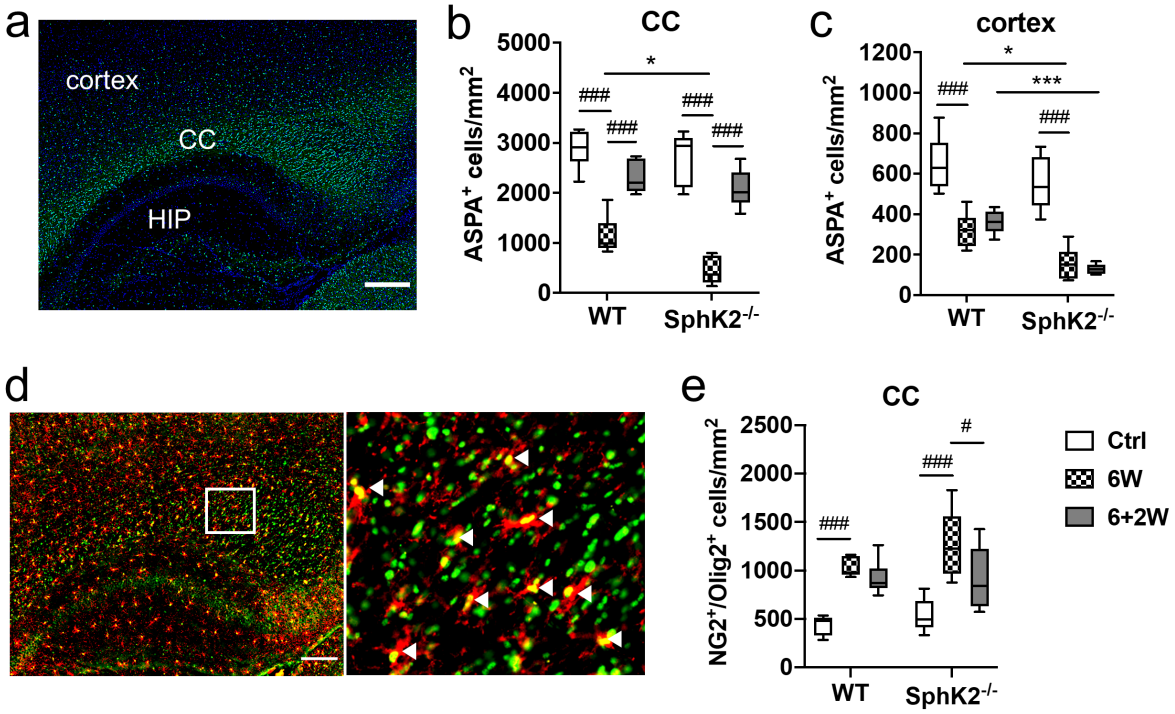

a
cortex
CC
HIP
b
CC
ASPA+ cells/mm2
5000
4000
3000
2000
1000
0
*
###
###
###
###
WT
SphK2-/-
c
cortex
ASPA+ cells/mm2
1200
1000
800
600
400
200
0
*
***
###
###
WT
SphK2-/-
d
e
CC
NG2+/Olig2+ cells/mm2
2500
2000
1500
1000
500
0
#
###
###
WT
SphK2-/-
Ctrl
6W
6+2W

Figure 4

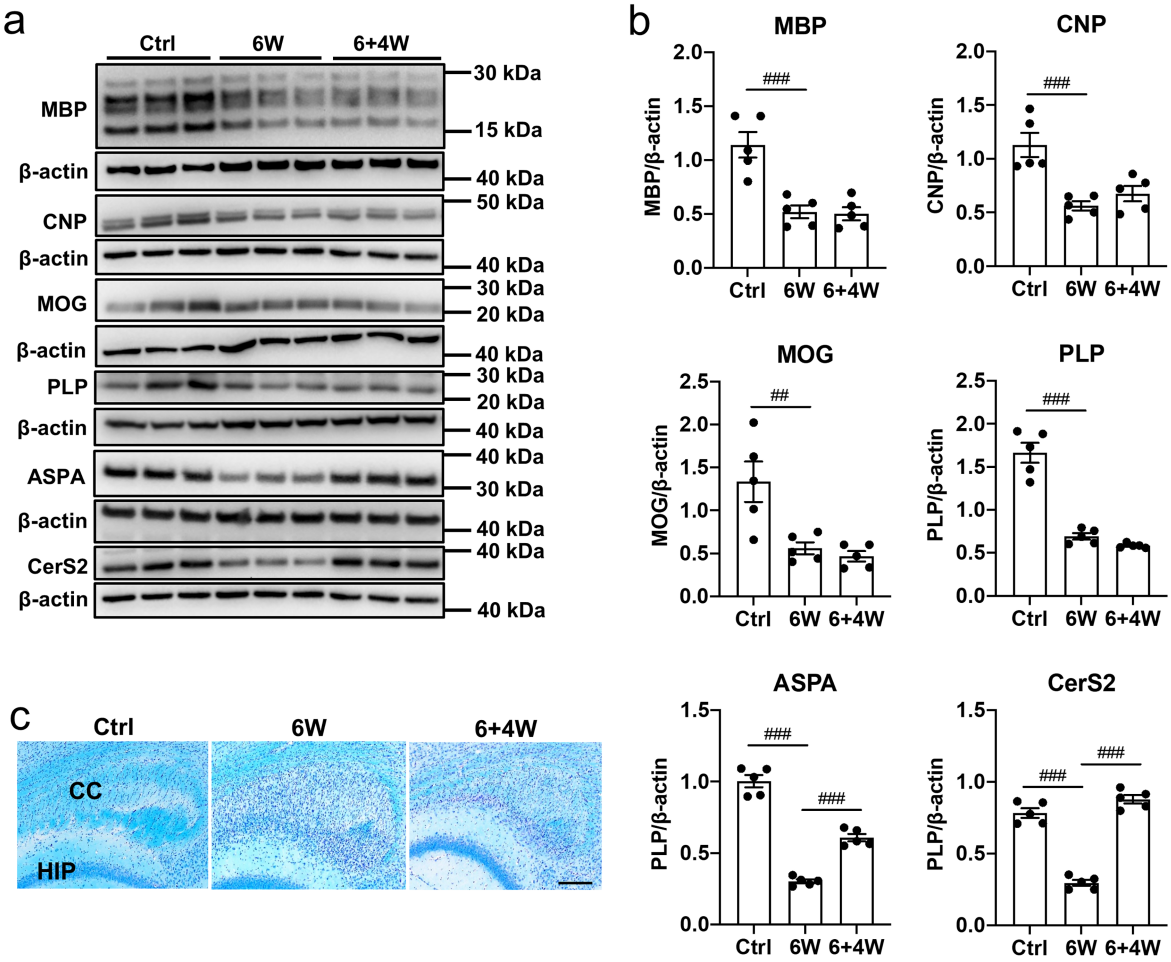
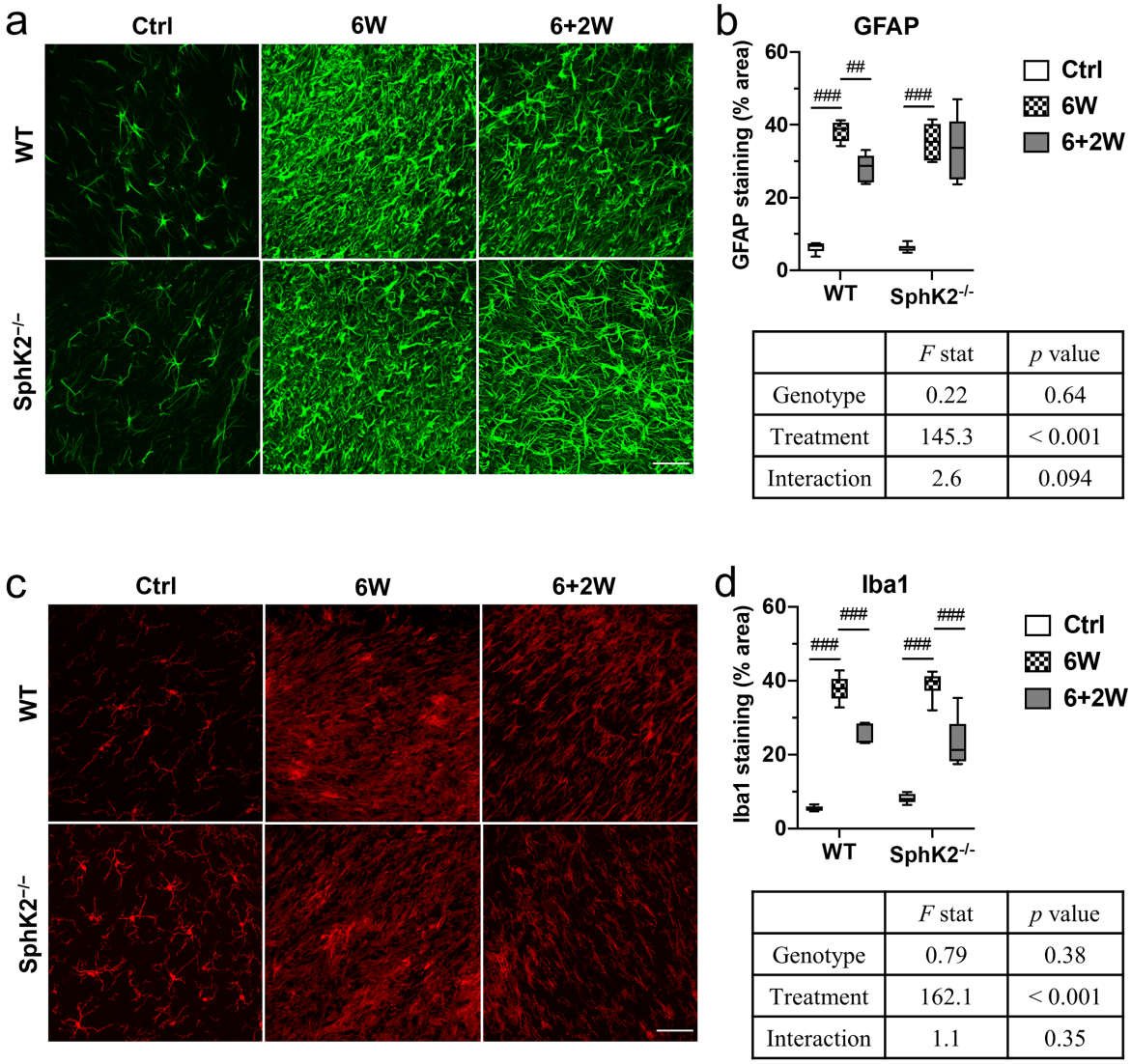

a
Ctrl
6W
6+4W
MBP
β-actin
CNP
β-actin
MOG
β-actin
PLP
β-actin
ASPA
β-actin
CerS2
β-actin
30 kDa
15 kDa
40 kDa
50 kDa
40 kDa
30 kDa
20 kDa
40 kDa
30 kDa
20 kDa
40 kDa
40 kDa
30 kDa
40 kDa
40 kDa
40 kDa
b
MBP
MBP/β-actin
CNP
CNP/β-actin
MOG
MOG/β-actin
PLP
PLP/β-actin
ASPA
PLP/β-actin
CerS2
PLP/β-actin
Ctrl
6W
6+4W
c
Ctrl
6W
6+4W
CC
HIP

Figure 5

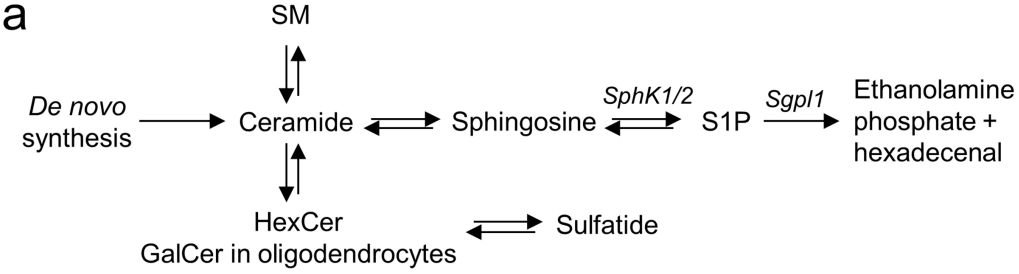


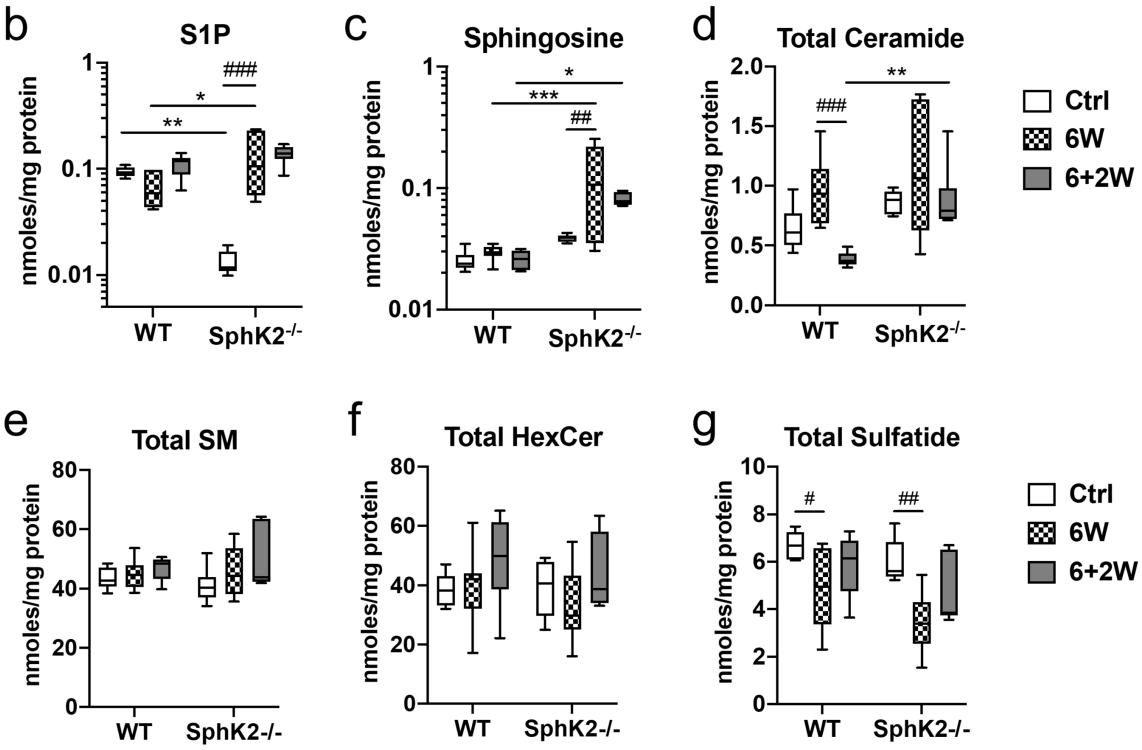
	F stat	*p* value
Genotype	0.22	0.64
Treatment	145.3	< 0.001
Interaction	2.6	0.094

	F stat	*p* value
Genotype	0.79	0.38
Treatment	162.1	< 0.001
Interaction	1.1	0.35

Figure 6

a

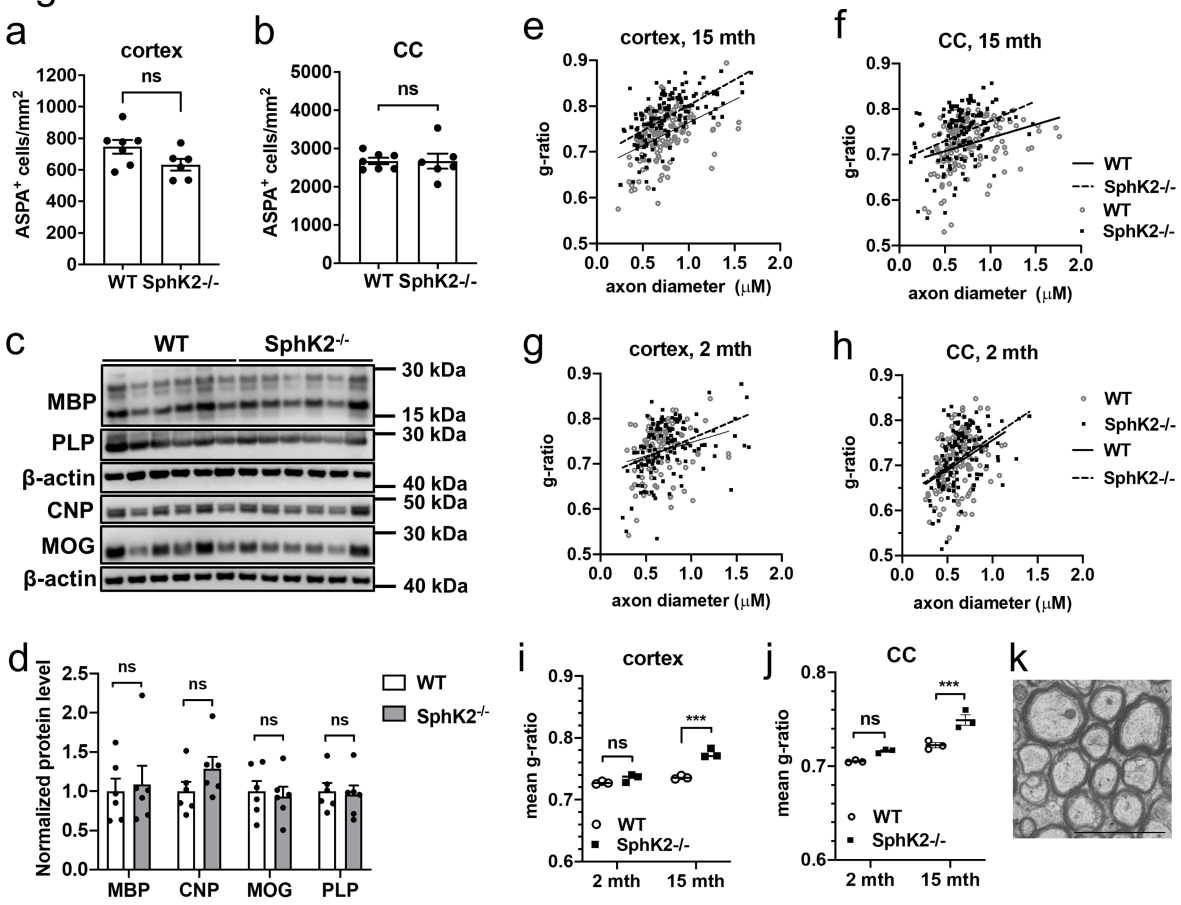




h

	Genotype		Treatment		Interaction	
	F stat	*p* value	*F* stat	*p* value	*F* stat	*p* value
S1P	0.05	0.81	**11.4**	**0.0002**	**12.0**	**0.0001**
Sphingosine	**20.2**	**0.00008**	**4.3**	**0.022**	**3.5**	**0.041**
Ceramide	**11.6**	**0.002**	**7.5**	**0.002**	1.7	0.19
Sulfatide	**7.0**	**0.012**	**10.5**	**0.0003**	0.21	0.81
HexCer	0.78	0.38	2.5	0.098	0.28	0.76
SM	0.02	0.89	2.5	0.098	0.44	0.64

Figure 7



	cortex		CC	
	F	*p*	*F*	*p*
Genotype	59.8	< 0.001	34.9	< 0.001
Age	65.9	< 0.001	61.7	< 0.001
Interaction	27.0	< 0.001	6.1	0.039

	Mean ± SEM (pmoles/mg protein)						Two-way ANOVA								
	Ctrl		6W		6+2W		Interaction			Genotype			Treatment		
	WT	SphK2$^{-/-}$	WT	SphK2$^{-/-}$	WT	SphK2$^{-/-}$	F	*p*	*q*	F	*p*	*q*	F	*p*	*q*
Sph 18:1	25.2 ± 2	38.7 ± 1.1	29.2 ± 1.6	124 ± 40.2 ***	25.8 ± 1.6	81.2 ± 3.5 *	3.53	4.1E-02	2.2E-01	20.20	7.7E-05	2.9E-03	4.27	2.2E-02	2.9E-02
S1P 18:1	93.8 ± 4.2	13.3 ± 1.4 **	67.2 ± 9	130.1 ± 34.5 *	110.1 ± 9.1	136.2 ± 10.3	12.00	1.1E-04	4.3E-03	0.06	8.1E-01	8.6E-01	11.36	1.7E-04	3.5E-04
Cer (d18:1/16:0)	64.4 ± 6.9	58.4 ± 6.9	132.9 ± 30.1	106.9 ± 19.7	38.4 ± 3.4	55.1 ± 5.2	1.00	3.8E-01	6.0E-01	0.16	6.9E-01	8.0E-01	12.56	8.2E-05	1.8E-04
Cer (d18:1/18:0)	509.9 ± 64.1	753 ± 33.9	730.8 ± 75.5	928.6 ± 197.9	311 ± 21.2	795.1 ± 86 **	1.48	2.4E-01	4.4E-01	16.74	2.5E-04	4.0E-03	4.91	1.3E-02	1.9E-02
Cer (d18:1/20:0)	1.6 ± 0.2	2.4 ± 0.7	2.9 ± 0.6	4.8 ± 0.9	2.7 ± 0.6	7.5 ± 2 **	2.02	1.5E-01	3.6E-01	8.73	5.7E-03	3.1E-02	4.67	1.6E-02	2.2E-02
Cer (d18:1/22:1)	8.6 ± 1.2	8.3 ± 1.1	7 ± 1.6	3.5 ± 1	2 ± 0.4	2 ± 0.6	1.79	1.8E-01	3.9E-01	2.21	1.5E-01	2.3E-01	19.87	1.9E-06	1.0E-05
Cer (d18:1/24:0)	0.7 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	0.9 ± 0.2	1.62	2.1E-01	4.2E-01	0.45	5.1E-01	6.0E-01	0.40	6.7E-01	6.7E-01
Cer (d18:1/24:1)	58 ± 5.8	45 ± 5.9	94.4 ± 14.1	75.6 ± 8.2	31.2 ± 3.2	44.5 ± 6.5	2.36	1.1E-01	3.5E-01	0.86	3.6E-01	4.4E-01	18.38	3.9E-06	1.7E-05
HexCer (d18:1/16:0)	428.5 ± 13.1	422.5 ± 37	389.4 ± 13.6	427.6 ± 29.8	401.4 ± 15.8	504.2 ± 44.7 *	1.93	1.6E-01	3.6E-01	3.81	5.9E-02	1.2E-01	1.33	2.8E-01	3.0E-01
HexCer (d18:1/18:0)	2652.4 ± 62.3	2960.2 ± 160.9	2747.7 ± 302.1	3289 ± 341.2	3503.4 ± 260.4	5588.8 ± 436.5 ***	5.51	8.4E-03	8.3E-02	16.06	3.2E-04	4.0E-03	21.30	1.0E-06	1.0E-05
HexCer (d18:1/18:0-OH)	739.3 ± 43	994.3 ± 114.2	858.3 ± 137.3	1037.2 ± 170.8	1140.1 ± 142.6	2181.4 ± 270.6 ***	4.30	2.2E-02	1.5E-01	12.84	1.0E-03	8.0E-03	14.30	3.1E-05	9.1E-05
HexCer (d18:1/20:0)	382.4 ± 22.7	421.8 ± 55.2	413.6 ± 66.2	428.6 ± 66.4	504.8 ± 61.9	675.4 ± 74.9	0.96	3.9E-01	6.0E-01	2.16	1.5E-01	2.3E-01	5.81	6.7E-03	1.0E-02
HexCer (d18:1/22:0)	430.8 ± 48.4	587.5 ± 126.7	835.4 ± 156	993.1 ± 187.3	1127.8 ± 174.8	1955.2 ± 248.7 **	2.57	9.1E-02	3.5E-01	7.02	1.2E-02	3.9E-02	17.98	4.7E-06	1.7E-05
HexCer (d18:1/22:0-OH)	464.1 ± 52.1	941 ± 295.3	978.4 ± 205.8	1153.2 ± 291.7	1345.6 ± 270.3	2150.3 ± 325.5	0.75	4.8E-01	6.4E-01	5.09	3.1E-02	7.8E-02	8.39	1.1E-03	2.0E-03
HexCer (d18:1/22:1)	2129.8 ± 114	2110.4 ± 162.1	1461.6 ± 182.6	993.8 ± 164.6	1570.1 ± 118.9	1151.8 ± 123.1	1.33	2.8E-01	4.6E-01	6.35	1.7E-02	4.8E-02	20.38	1.5E-06	1.0E-05
HexCer (d18:1/22:1-OH)	934.5 ± 52.2	1247 ± 108.3	721.3 ± 93	587.8 ± 107.1	797.9 ± 76	669.3 ± 70.5	4.19	2.4E-02	1.5E-01	0.06	8.1E-01	8.6E-01	13.65	4.4E-05	1.1E-04
HexCer (d18:1/24:0)	376.3 ± 48.7	559.1 ± 142	862.7 ± 139.1	1049.8 ± 253	1280 ± 236.2	2264.5 ± 349.7 **	2.17	1.3E-01	3.5E-01	5.87	2.1E-02	5.7E-02	17.20	6.9E-06	2.2E-05
HexCer (d18:1/24:0-OH)	287.9 ± 31.7	542.2 ± 160.2	680.5 ± 135.2	643.6 ± 185.2	878.2 ± 170.2	1207 ± 212.6	0.70	5.1E-01	6.4E-01	1.82	1.9E-01	2.6E-01	7.53	2.0E-03	3.2E-03
HexCer (d18:1/24:1)	25932.9 ± 1741	23620 ± 2359.1	25015.6 ± 3145.3	18423.7 ± 3269.8	30242.1 ± 3075.8	20850.5 ± 2145.8 *	0.83	4.5E-01	6.3E-01	7.28	1.1E-02	3.7E-02	1.09	3.5E-01	3.7E-01
HexCer (d18:1/24:1-OH)	3662.4 ± 308.3	4591.2 ± 650.4	4349.7 ± 687.5	3923.4 ± 730.1	5219.4 ± 715.9	4491.4 ± 534.4	0.92	4.1E-01	6.0E-01	0.02	8.9E-01	8.9E-01	0.90	4.1E-01	4.3E-01
SM (d18:1/16:0)	2136.4 ± 71.9	2022.4 ± 85.4	3354 ± 296	3828.1 ± 294	3341.8 ± 97.5	4119.9 ± 333 *	1.93	1.6E-01	3.6E-01	4.09	5.1E-02	1.2E-01	30.52	2.6E-08	5.1E-07
SM (d18:1/18:0)	11535.3 ± 326.7	11291.4 ± 415.2	12360.6 ± 688	12785.5 ± 877.1	13110.6 ± 377.3	15196.9 ± 1055.2	1.58	2.2E-01	4.2E-01	1.81	1.9E-01	2.6E-01	8.19	1.3E-03	2.2E-03
SM (d18:1/20:0)	1246.7 ± 84	1069.9 ± 65.9	1437.7 ± 55.5	1424.9 ± 106.3	1574.4 ± 48.7	1396.9 ± 109.6	0.69	5.1E-01	6.4E-01	3.47	7.1E-02	1.4E-01	9.23	6.3E-04	1.2E-03
SM (d18:1/22:0)	1644.7 ± 179.3	1423.2 ± 123.1	2454.1 ± 223.8	3095.6 ± 323.7	2350.2 ± 109.7	2949.6 ± 244.2	2.56	9.2E-02	3.5E-01	3.94	5.5E-02	1.2E-01	20.15	1.7E-06	1.0E-05
SM (d18:1/22:1)	3273.7 ± 339	3823.6 ± 467	3155.8 ± 320.4	4515.4 ± 696.6	2379.7 ± 113	3056.3 ± 248.4	0.65	5.3E-01	6.4E-01	7.76	8.7E-03	3.7E-02	5.00	1.2E-02	1.8E-02
SM (d18:1/24:0)	595 ± 58.5	568 ± 52.2	990 ± 83.5	1369.4 ± 154.2	1184.6 ± 76.6	1665.9 ± 170 **	2.88	7.0E-02	3.3E-01	9.49	4.1E-03	2.6E-02	30.38	2.7E-08	5.1E-07
SM (d18:1/24:1)	22954.7 ± 723.5	20763.1 ± 1486.2	20862.9 ± 1339.7	18576 ± 1600.6	22772.3 ± 933.9	20652.3 ± 1803.5	0.00	1.0E+00	1.0E+00	3.87	5.7E-02	1.2E-01	1.50	2.4E-01	2.6E-01
ST (d18:1/16:0)	12.3 ± 1	9.6 ± 0.8	11.6 ± 0.9	11.6 ± 0.8	13.5 ± 0.8	20.7 ± 3.2 **	5.46	8.8E-03	8.3E-02	1.29	2.6E-01	3.3E-01	9.48	5.4E-04	1.1E-03
ST (d18:1/18:0)	425.7 ± 11.1	421.4 ± 28.4	354.7 ± 31.9	296.3 ± 26.4	410.3 ± 24.8	572.3 ± 54.1 **	6.33	4.6E-03	8.3E-02	1.48	2.3E-01	3.0E-01	13.06	6.2E-05	1.5E-04
ST (d18:1/20:0)	108.1 ± 7.1	100.1 ± 5.9	79.3 ± 12.3	59 ± 11.6	93 ± 8.7	85.7 ± 10.8	0.27	7.6E-01	8.1E-01	2.14	1.5E-01	2.3E-01	5.93	6.2E-03	9.8E-03
ST (d18:1/22:0)	233.5 ± 13.7	225.4 ± 16	190.1 ± 36.6	157 ± 26	233.9 ± 23.4	257 ± 35.4	0.54	5.9E-01	6.7E-01	0.07	7.9E-01	8.6E-01	3.76	3.3E-02	4.1E-02
ST (d18:1/22:0-OH)	56.1 ± 4.5	78.5 ± 13	57.4 ± 11.5	48.4 ± 7.2	67 ± 9.2	85.3 ± 10.8	1.42	2.5E-01	4.4E-01	1.68	2.0E-01	2.8E-01	2.87	7.0E-02	8.4E-02
ST (d18:1/22:1)	243.4 ± 11	214.3 ± 14.4	150.1 ± 21.1	108.6 ± 21.4	185.3 ± 12.1	139.8 ± 15.1	0.13	8.8E-01	9.0E-01	8.36	6.7E-03	3.2E-02	18.01	4.6E-06	1.7E-05
ST (d18:1/22:1-OH)	99.2 ± 6.3	107.4 ± 5.6	63.8 ± 8.1	51.9 ± 9.1	85.4 ± 7.9	60.7 ± 6.4	2.44	1.0E-01	3.5E-01	2.40	1.3E-01	2.3E-01	17.91	4.9E-06	1.7E-05
ST (d18:1/24:0)	308.3 ± 21.9	331.8 ± 34.3	325.7 ± 59.7	282.4 ± 49.9	412 ± 47.3	449.3 ± 59.1	0.39	6.8E-01	7.4E-01	0.02	8.9E-01	8.9E-01	4.16	2.4E-02	3.1E-02
ST (d18:1/24:0-OH)	34.1 ± 4.2	48.4 ± 11.5	39.7 ± 7.8	39.4 ± 8.6	47.2 ± 7.5	65.4 ± 10.6	0.63	5.4E-01	6.4E-01	2.28	1.4E-01	2.3E-01	2.36	1.1E-01	1.3E-01
ST (d18:1/24:1)	4793.5 ± 187.8	4088.5 ± 288.7	3142.6 ± 427.1	2194.4 ± 356.5	4042.1 ± 300.2	2707 ± 307.1 *	0.48	6.2E-01	6.9E-01	13.87	7.1E-04	6.7E-03	13.97	3.7E-05	1.0E-04
ST (d18:1/24:1-OH)	380.8 ± 15.9	381.9 ± 29.7	252.4 ± 35.1	182.7 ± 28.1	297.2 ± 25.1	190 ± 16.2 *	2.16	1.3E-01	3.5E-01	7.42	1.0E-02	3.7E-02	21.09	1.1E-06	1.0E-05

Table 2. Levels of individual sphingolipid species in CC. Results of two-way ANOVA show both unadjusted *p* values, and those adjusted for multiple comparisons (*q* values) using the Benjamini-Hochberg correction. Bonferonni post-test was used to compare WT and SphK2$^{-/-}$ groups within each treatment group: *$p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. Sph: sphingosine; Cer: ceramide; HexCer, hexosylceramide; SM, sphingomyelin, ST, sulfatide.