Mass spectrometry imaging and LC/MS reveal decreased cerebellar phosphoinositides in Niemann-Pick type C1-null mice.

Koralege C. Pathmasiri,* Melissa R. Pergande,* Fernando Tobias,* Rima Rebiai,†,§ Avia Rosenhouse-Dantsker,* Ernesto R. Bongarzone,§ and Stephanie M. Cologna†,*,*

Department of Chemistry,* Laboratory of Integrated Neuroscience,† and Department of Anatomy and Cell Biology,§ University of Illinois at Chicago, Chicago, IL.

ORCID IDs: 0000-0003-2847-8551 (K.C.P); 0000-0003-1197-4057 (M.R.P); 0000-0002-4085-4188 (F.T.); 0000-0002-3541-3961 (S.M.C.)

Abstract  Niemann-Pick disease type C1 (NPC1) is a lipid storage disorder in which cholesterol and glycosphingolipids accumulate in late endosomal/lysosomal compartments because of mutations in the NPC1 gene. A hallmark of NPC1 is progressive neurodegeneration of the cerebellum as well as visceral organ damage; however, the mechanisms driving this disease pathology are not fully understood. Phosphoinositides are phospholipids that play distinct roles in signal transduction and vesicle trafficking. Here, we utilized a consensus spectra analysis of MS imaging data sets and orthogonal LC/MS analyses to evaluate the spatial distribution of phosphoinositides and quantify them in cerebellar tissue from Npc1-null mice. Our results suggest significant depletion of multiple phosphoinosidade species, including PI, PIP, and PIP$_2$, in the cerebellum of the Npc1-null mice in both whole-tissue lysates and myelin-enriched fractions. Additionally, we observed altered levels of the regulatory enzyme phosphatidylinositol 4-kinase type 2a in Npc1-null mice. In contrast, the levels of related kinases, phosphatases, and transfer proteins were unaltered in the Npc1-null mouse model, as observed by Western blot analysis. Our discovery of phosphoinositide lipid biomarkers for NPC1 opens new perspectives on the pathophysiology underlying this fatal neurodegenerative disease.—Pathmasiri, K. C., M. R. Pergande, F. Tobias, R. Rebiai, A. Rosenhouse-Dantsker, E. R. Bongarzone, and S. M. Cologna. Mass spectrometry imaging and LC/MS reveal decreased cerebellar phosphoinositides in Niemann-Pick type C1-null mice. J. Lipid Res. 2020, 61: 1004–1013.

Supplementary key words  cholesterol • neurodegeneration • phosphoinositide signaling • bisphosphate • lysosomal storage disorder • phospholipid • signal transduction • genetic metabolic disorder

Phospholipids play key roles in a range of cellular processes, including energy storage, structural maintenance, protein synthesis and degradation, and cellular signaling. Among them, phosphoinositides are important mediators of signal transduction and vesicle trafficking (1). Phosphoinositides can be monophosphorylated (PIP), bisphosphorylated (PIP$_2$), or triphosphorylated (PIP$_3$), with each species expressing in specific cellular locations and having unique cellular functions (2, 3). Alterations in the synthesis and metabolism of phosphoinositides have been implicated in a number of diseases, including neurodegenerative disorders (4).

Niemann-Pick disease type C1 (NPC1) is a fatal, genetic, neurodegenerative, lysosomal storage disorder. Biochemical manifestations of NPC1 include the accumulation of cholesterol and glycosphingolipids in the late endosomal/lysosomal compartments (5). This accumulation, in turn, leads to a number of secondary events, including oxidative stress (6), mitochondrial dysfunction (7), and progressive degeneration of cerebellar Purkinje neurons (8). There is currently no US Food and Drug Administration-approved therapy for NPC1, highlighting the need to determine the mechanisms driving cell death. To this end, the Npc1-null mouse model, hereafter referred to as Npc1$^{-/-}$ mice, recapitulates the human disease. The life span of Npc1$^{-/-}$ mice includes a presymptomatic time of up to 3 weeks of age that is followed by the development of tremors at 5 weeks and ataxia at 7 weeks, with death occurring at approximately 3 months of age (9, 10). A progressive loss of cerebellar Pur-
kinje neurons is observed in this model and supports clinical phenotypes (11).

Several lipid analyses of NPC1 models have given rise to our current knowledge about disruptions in lipid homeostasis. In view of the lysosomal accumulation of cholesterol and glycosphingolipids that occurs in the disease, Fan et al. (12) profiled a series of sphingolipids in multiple tissues from the Npc1 mouse model showing alterations, including an elevation of the gangliosides GM2 and GM3 and decreased sulfatides in whole brain tissues. Other lipid studies identified lysosphingolipid biomarkers (13) and altered oxysterols (14) in NPC1. Phospholipid alterations in NPC1, however, have been less explored (15). Notably, decreased PIP2 has been implicated in a point mutation mouse model of NPC1 contributing to ion channel dysfunction (16). However, it remains unknown whether multiple phosphoinositide levels are also altered in the Npc1-null mouse model that completely lacks the gene. Interestingly, our recent proteomic studies have revealed that the enzymes responsible for these important signaling lipids are altered in the null mouse model at preterminal ages (17, 18).

Thus, to further investigate lipid distribution patterns in cerebellar tissue from control (Npc1+/+) and Npc1−/− mice, identify new biomarkers, and interrogate altered pathways in NPC1, we used mass spectrometry imaging (MSI). We recently used MSI to study NPC1 ganglioside distributions and developed an algorithm to evaluate biological and technical MSI data sets in the cerebellum of symptomatic Npc1−/− animals (19). In another study, a point mutation NPC1 mouse model was used to develop a combined infrared microscopy-MSI approach, but lipid mapping was not extensively performed (20). More recently, we have shown that ceramides exhibit patterned alterations in the cerebellum of Npc1−/− mice via MSI (21). Herein, we show that PIP2 and PIP are decreased in cerebellar tissue from the Npc1 mouse model as early as 3 weeks of age, whereas a decrease in PI is observed at a later stage of the disease progression by LC/MS. MSI reveals overall decreased levels of PIP2 and PI, consistent with LC/MS data; however, no specific regional differences are observed. These findings were further interrogated and revealed the stereochemistry of PIP2, as well as significant alterations of PIP and PIP2 in myelin-enriched fractions. The data suggest that phosphoinositides and their respective regulatory enzymes may be biomarkers of NPC1. Hence, phosphoinositide signaling pathways may constitute novel therapeutic targets for NPC1.

MATERIALS AND METHODS

Materials

Trimethylsilyl diazomethane, acetonitrile (LC/MS grade), formic acid (reagent grade), ammonium formate (reagent grade), chloroform (LC/MS grade), methanol (LC/MS grade), piperidine (reagent plus grade), Tergitol type NP-40 and Trizma base (reagent grade) DTT, PMSF, and sucrose were obtained from Sigma-Aldrich. EDTA was obtained from Thermo Fisher Scientific, and hydrochloric acid was obtained from VMR Chemicals. Lipid standards were purchased from Avanti Polar Lipids. All reagents were used as supplied unless otherwise noted.

Animal model

All experiments were performed according to University of Illinois at Chicago Institutional Animal Care and Use Committee-approved protocols. Balb/c Npc1+/+ (Npc1−/−) mice were obtained from The Jackson Laboratory (RRID:IMSR JAX:003092), and a breeding colony was maintained by our laboratory. Genotyping was performed using PCR as previously reported (22). At 3, 7, and 9 weeks of age, control (Npc1+/+) and null (Npc1−/−) mice were euthanized via CO2 asphyxiation followed by decapitation. Whole brain tissue was dissected, immediately frozen on dry ice to maintain spatial integrity, and stored at −80°C.

Sample preparation for MSI experiments

Frozen, intact brain tissue was sectioned at −12°C using a Microm HM 525 cryostat (Thermo Fisher Scientific). Serial tissue sections of 10 μm thickness were thaw-mounted directly onto stainless-steel MALDI plates and stored at −80°C. Npc1−/− and age-matched control tissue sections were mounted on the same MALDI plate in a pairwise manner for analysis in the same experiment for reliable data comparison. Prior to analysis, the plate was dried under vacuum for 2 min to remove residual moisture. The plate was then submerged in cold 50 mM ammonium formate for 20 s, dried again, and weighed. This was followed by matrix application using a home-built sublimation apparatus consisting of a rough pump, cold trap, pressure gauge, temperature probe, sand bath, and sublimation chamber. Here, 9-aminoacridine was dissolved in acetone (40 mg/5 ml) and transferred to the bottom of the sublimation chamber. The residual solvent was evaporated via a steady stream of nitrogen gas, resulting in the formation of a uniform coating onto the glass bottom. Next, the plate was attached to the flat bottom of the cold finger using conductive copper tape. The sand bath temperature was set to 150°C and the trap was filled with ice water. During the sublimation process (4.5 min), the internal vacuum was maintained at 80 mTorr. The plate was weighed again postsublimation to obtain the matrix density (0.18-0.22 mg/cm2).

MSI experiments

MSI was performed using a 4800 Plus MALDI TOF/TOF Analyzer (Sciex) equipped with a 200 Hz Nd:YAG (355 nm) pulsed laser. Data were acquired in the negative-ion reflectron mode, in which red phosphorus was used for external mass calibration (23). The number of laser shots per pixel was set to 50, and the raster distance between each pixel was set to 50 μm using 4800 Imaging Tool v3.2 (https://ms-imaging.org/wp/4000-series-imaging). The acquired data were processed using MSIReader, in which regions of interest were limited to the cerebellum (24). MS images were normalized by dividing the intensity by the total ion current (TIC). Biological and technical replicate analysis was carried out using averaged region-of-interest mass spectrum data in R-Programming Language by normalizing the null mouse mass spectrum to the age-matched control as previously described (19). Lipid assignments were made by comparing accurate mass measurements to the LIPID MAPS database, with an allowance of tolerance of ±0.05 m/z. On-tissue MS/MS data were acquired to yield signature fragment ions of each lipid species to increase the confidence in these initial lipid assignments. When possible, MS/MS spectra were compared with commercially available standards.

Extraction of phosphoinositide species

Cerebellar tissue from Npc1+/+ and Npc1−/− animals at 3 and 9 weeks of age (N = 3 for each genotype) was homogenized in
ice-cold lysis buffer (50 mM Trizma base, 0.1% NP-40, and 5 mM EDTA) to extract phosphoinositides from the whole cerebellum. Cerebellar tissue from Npc1+/+ and Npc1−/− animals at 7 weeks of age (N = 3 for each genotype) was homogenized in 0.3 M sucrose solution [20 mM Tris base (pH 7.5), 1 mM EDTA, 1 mM DTT, 100 µM PMSF] using a pestle homogenizer, and myelin was isolated as previously described (25). Homogenized cerebellar tissue and isolated myelin were lysed by probe sonication (four 7 s pulses at 40% amplitude). Protein concentration was measured using the Pierce BCA Protein Assay (Thermo Fisher Scientific). Organic extraction of phosphoinositides was performed via an acidified Bligh and Dyer method of tissue lysates (500 µg protein equivalent for whole cerebellum and 50 µg protein equivalent from isolated myelin) (26–28). The resulting extracts were phosphomethyl-derivatized to increase ionization efficiency in the positive-ion mode as previously described (29, 30). For the direct analysis of phosphoinositides without methyl derivatization, an acidified Bligh and Dryer extraction was performed using the same protocol as described above. The methyl-derivatized extracts were dried at 30°C and resuspended in 50 µl methanol prior to analysis. The nonderivatized samples were dried at 30°C and resuspended in 50 µl methanol containing 5 mM piperidide.

**Lipid extraction for cardiolipin analysis**

Cerebellar tissue from Npc1+/+ and Npc1−/− animals at 3 and 9 weeks of age (N = 3 for each genotype) was homogenized in ice-cold lysis buffer (1× PBS containing protease and phosphatase inhibitors). Protein concentration was measured using the Pierce BCA Protein Assay (Thermo Fisher Scientific). Organic extraction of lipids was performed via the Folch lipid extraction method (31) from a 200 µg protein equivalent of the lysates. The organic layer resulting from the lipid extraction was dried at 30°C and resuspended in 200 µl methanol-chloroform (3:1, v/v).

**LC/MS analysis of methyl-derivatized phosphoinositides**

Lipid separation was performed using an Agilent 1290 Infinity II UHPLC system outfitted with an Agilent EclipsePlus C18 RRHD column (2.1 × 100 mm, 1.8 µm). The column was maintained at 50°C and operated at a flow rate of 0.5 ml/min. Mobile phases consisted of (A) 60:40 (v/v) acetonitrile-water with 0.1% formic acid and 10 mM ammonium formate and (B) 90:10 (v/v) isopropanol-acetonitrile with 0.1% formic acid and 10 mM ammonium formate. From each biological sample, 5 µl was injected and analyzed in technical triplicate. The LC gradient was as follows: 0–2 min 48% (B), 2–10 min 48% (B) increased to 82% (B), 10–14.5 min 82% (B) increased to 99% (B), and 14.5–20 min held at 99% (B). Data-dependent acquisition was performed using an Agilent 6545 Q-TOF mass spectrometer in the positive-ion mode. The MS and MS/MS parameters were as follows: gas temperature (200°C), drying gas (20:30:50, v/v/v) acetonitrile-methanol-isopropanol with 0.05 mM ammonium fluoride and 10 mM ammonium acetate and (B) 20:30:50 (v/v/v) acetonitrile-methanol-isopropanol with 0.05 mM ammonium fluoride and 10 mM ammonium acetate. From each sample 2 µl was injected. Data were acquired using an Agilent 6545 Q-TOF in the negative-ion mode with the m/z range set to 200–1700. A pooled sample was analyzed in negative-ion polarity using the iterative MS/MS mode function with a fixed collision energy of 25 eV. Iterative MS/MS data were searched in Agilent Lipid Annotator software for lipid identification. Targeted MS/MS was performed for the identified cardiolipins (CLs) using the Lipid Annotator results and carried out at a fixed collision energy of 65 eV.

Data were analyzed manually in Agilent MassHunter software using retention time and MS/MS fragmentation matching of phosphoinositide (i.e., PIP and PIP2) standards prepared following the same method used for lipid extracts. Graphical data and statistical analyses were carried out using GraphPad Prism.

**Western blot analysis of murine cerebellar tissue**

Control and null-mouse lysates for each of the animals at 3 and 9 weeks of age were analyzed (N = 3 for each genotype). Tissue was homogenized in RIPA buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate] for cellular lysis and protein extraction. Protein concentration was determined via the BCA assay (Thermo Fisher Scientific). Samples were heated in a Laemmli buffer containing dithiothreitol at 70°C for 10 min before loading onto a 12% NuPage Bis-Tris gel (Novex; Life Technologies). Proteins were separated by SDS-PAGE (120 V for ~120 min) and transferred to a nitrocellulose membrane via an Invitrogen blotting apparatus (Thermo Fisher Scientific) at 10 V for 30 min. Membranes were blocked in a solution of 5% (w/v) dry milk and 0.1% (v/v) PBS-T overnight before the addition of a primary antibody [Pip5K1α (Cell Signaling Technologies), 1:1000; PhK2α (Thermo Fisher Scientific), 1:500; Pten (AbCam ab170941), 1:1000; Inpp5e (AbCam ab230642), 1:100] followed by the addition of the secondary antibody (1:2500 dilution) in 5% (w/v) milk PBS-T for 1 h. Excess secondary antibody was removed by washing with PBS-T three times before protein bands were visualized by incubation with West Pico blotting substrate (Thermo Fisher Scientific) and imaging on an Azure e300 imaging system. Western blot signals were compared via densitometry analysis using ImageJ (32).

**RESULTS**

NPC1 is a lysosomal storage disorder characterized by progressive cerebellar neurodegeneration, particularly a patterned loss of Purkinje neurons (11). In this study, we used the Npc1-null mouse model to investigate changes that occur in phosphoinositide levels and distribution in parallel to progressive neurodegeneration in whole cerebellar tissue and in myelin-enriched fractions. Further, we investigated the source of altered PIP2 by evaluating the enzymes responsible for the synthesis of the different PIP2 structural isomers.
MALDI-MSI reveals altered phosphoinositides in Npc1−/− cerebellum

To determine the lipid profile of the cerebellum throughout disease progression, we performed MALDI-MSI of the cerebellum at 3, 7, and 9 weeks of age in Npc1+/− and Npc1−/− mice. Replicate analysis was conducted incorporating both technical and biological replicates, and consensus spectra were generated for both genotypes at each time point (Fig. 1A–C) using a tool that we have recently developed (19).

The consensus spectrum is a normalized and filtered mass spectrum. It illustrates the top 40 most abundant peaks (user-defined) for the whole cerebellum, representing data from multiple biological and technical replicates. Several interesting observations were made by evaluating the consensus spectra. First, among the peaks observed in the m/z 1300–1500 range, a large increase in the normalized abundance of m/z 1382.8 and m/z 1410.8 in the Npc1-null mice compared with the control mice was seen at all three time points. Accurate mass measurement matching and MS/MS fragmentation with database searching identified m/z 1382.8 as GM2(d36:1) and 1410.8 as GM2(d38:1). This is consistent with previous reports of ganglioside increases in NPC1 (21, 33). On tissue MS/MS spectra are provided in supplemental Figs. S1 and S2 for m/z 1382.8 and 1410.8, respectively, indicating the sialic acid m/z 290.5 diagnostic fragment ion. A slight increase in the normalized intensity was observed for m/z 1179.8 in the Npc1-null mouse data set at 7 and 9 weeks. The MS/MS spectrum of m/z 1179.8 (supplemental Fig. S3) also indicated the presence of a sialic acid moiety with an accurate mass corresponding to GM3(d36:1). We observed no difference in the abundance for the peaks at m/z 1544.4 and 1572.6; these peaks were assigned as GM1(d36:1) and GM1(d38:1), respectively (supplemental Figs. S4, S5). This observation of GM2 and GM3 accumulation in the Npc1-null mouse cerebellum is consistent with our previous findings using 7 week old animals and further illustrates the consistency of ganglioside accumulation throughout disease progression (19, 21).

Within the m/z 1400–1500 range, several ions likely to be CL species displayed altered abundance values in the Npc1-null mouse model data set (Fig. 1, supplemental Fig. S6). However, these assignments are based only on accurate mass measurement, as on-tissue MS/MS spectra did not provide sequence-informative information. Thus, to confirm these assignments, we compared lipid extracts obtained from Npc1−/− cerebellar lysates at 3 and 9 weeks with the control using LC/MS (supplemental Fig. S7A–C). These studies confirmed the three CL species (MS/MS fragmentation shown in supplemental Fig. S8A–C) that we observed in the MALDI-MSI consensus spectra (Fig. 1). Among these identified species, CL (70:4) at m/z 1427.8 and CL (70:5) at m/z 1425.9 exhibited elevated levels in symptomatic Npc1−/− cerebellum at 9 weeks. Conversely, no change in these CL species was observed at the pre-symptomatic age of 3 weeks (supplemental Fig. S6). These observations further corroborate the trend shown in the 9 week MALDI-MSI consensus spectra (Fig. 1, supplemental Fig. S6). The third CL species identified in our LC/MS/MS studies, CL (74:8), did not display any difference at either age (supplemental Fig. S7C). Remarkably, CLs are known to interact with mitochondrial proteins for membrane stability as well as for their involvement in mitochondrial apoptosis (34). Because mitochondrial dysfunction and defective antioxidant defense has been reported in NPC1, alterations in CL levels in NPC1 may play an important role in these mitochondrial defects (35).

Most interestingly, consistent reduction in the normalized abundance of putatively assigned PIP2 at m/z 1045.5 was revealed by the consensus spectra (Fig. 1). Based on accurate mass, the total carbon to double bond ratio of the fatty acid chain was determined to be 38:4; however, with this method, the specific length and double bond position cannot be determined. The consensus spectra of Npc1−/− and Npc1+/− indicated an alteration in signal from the whole cerebellum, which led us to further inspect MS images of m/z 1045.5 at different time points (Fig. 2A). Depletion of PIP2 (38:4) localized to both the gray and white matter of the cerebellum and was observed for all three time points in which

**Fig. 1.** MALDI-MSI analysis of cerebellar tissue in the NPC1 mouse model reveals alterations in PIP2 (38:4). MALDI-MSI was performed on cerebellar tissue of age-matched Npc1−/− and Npc1+/− mice. Consensus spectra were generated for the acquired images using multiple biological (N) and technical (n) replicates at three time points of disease progression: (A) week 3 Npc1−/− N = 2, n = 5; week 3 Npc1+/− N = 3, n = 7; (B) week 7 Npc1−/− N = 3, n = 8; week 7 Npc1+/− N = 3, n = 9; and (C) week 9 Npc1−/− N = 3, n = 7; week 9 Npc1+/− N = 2, n = 4. Red is Npc1−/−, blue is Npc1+/−, and the boxed peak denotes the m/z corresponding to PIP2 (38:4) (m/z 1045.5).
the white matter showed the most drastic depletion of signal. To validate the molecular assignment of PIP2 (38:4), on-tissue tandem MS/MS was conducted that revealed diagnostic ions for the native PIP2 species that matched a commercial PI(4,5)P2 standard (supplemental Fig. S9).

In view of the multiplexing nature of MALDI-MSI, we next investigated the total monophosphate, PIP, and the precursor PI. The signal for PIP was not pronounced; however, a robust signal corresponding to PI (38:4) was observed (Fig. 2B). To validate the molecular assignment of PI (38:4), on-tissue MS/MS was performed (supplemental Fig. S10). In contrast to the abundant distribution of PIP2 (38:4) in white matter, its precursor, PI (38:4), was more abundant in cerebellar gray matter (an overlay image is shown in supplemental Fig. S11). MS images of each mouse genotype at 3 and 7 weeks suggested no significant alteration in PI (38:4) level in the Npc1−/− mouse; however, by 9 weeks, a depletion of PI (38:4) in Npc1−/− mice was observed relative to Npc1+/+

**LC/MS analysis reveals decreased phosphoinositides in Npc1+/+ and Npc1−/− mice cerebellum.**

To corroborate our initial finding of decreased PIP2 (38:4) in the Npc1−/−-null mouse model, we performed LC/MS to further investigate the altered levels of PIP2. We also determined whether other phosphoinositide species in cerebellar lysates were altered, as this remained unclear in our MSI studies. Using LC/MS methods developed with lipid standards, we first evaluated phosphoinositides with the most abundant fatty acyl chain composition (38:4), namely, PIP2 (38:4), PIP (38:4), and PI (38:4) (Fig. 3, supplemental Fig. S12). Using MS/MS fragmentation patterns of the above phosphoinositide species, we found that the most abundant phosphoinositides in murine cerebella contain acyl chains composed of arachidonic acid (20:4) and steric acid (18:0) (supplemental Fig. S13–S15). An analysis of the total content of these phosphoinositides revealed that not only PIP2 but also PIP and PI were significantly decreased in the cerebellum of Npc1−/− mice. Interestingly, decreased levels of PIP2 (20:4/18:0) and PIP (20:4/18:0) were observed at both the early (3 week) and late (9 week) time points of the disease (Fig. 3A, B). In contrast, a statistically significant decrease in PI (20:4/18:0) was observed only at the symptomatic 9 week time point (Fig. 3C), suggesting that PI (20:4/18:0) depletion occurs only after significant neurodegeneration and clinical phenotypes.

Considering these results only represent the (20:4/18:0) fatty acyl composition, we next investigated the potential of alterations in phosphoinositide levels in the Npc1-null model with other fatty acyl chains. To this end, we carried out an LC/MS analysis of additional phosphoinositides with a focus on other varying fatty acid compositions (supplemental Fig. S16), including structure elucidation using MS/MS spectra (supplemental Fig. S13–S15). Unlike PIP2 (20:4/18:0), both PIP2 (20:4/18:1) and PIP2 (20:4/16:0) were not detected at 3 weeks of age, and a decrease in these phosphoinositides compared with the
control was only observed at 9 weeks. In contrast, and similar to PIP (20:4/18:0), the monophosphate PIP (20:4/18:1) was decreased at both the presymptomatic and symptomatic time points. A similar trend was also obtained for PI (20:4/18:0) and PI (20:4/18:1). Both phosphoinositides were decreased only at the symptomatic time point. Conversely, PI (20:4/16:0) appeared to be slightly elevated in 9 week old Npc1−/− animals compared with Npc1+/+ animals. Together, these distinct trends suggest that the fatty acyl chain composition plays an important role in determining the impact of the NPC1 protein on phosphoinositide levels.

In view of the substantial differences observed in phosphoinositide abundance in the white matter of the cerebellum in the MALDI-MSI studies, we next analyzed the different phosphoinositides in isolated myelin fractions from 7 week old Npc1−/− and age-matched control mice (Fig. 4). The data confirmed the depletion of PIP2 (20:4/18:0) and PIP (20:4/18:0) in Npc1−/− cerebellar white matter (Fig. 4A, B). Interestingly, no differences were observed in PI (20:4/18:0) (Fig. 4C). Taken together, these results reveal alterations in phosphoinositide levels and distributions in the cerebella of Npc1−/− mice, suggesting that important phosphoinositide signaling pathways may be affected in the disease in both gray and white matter regions of the brain.

In the above studies, the analysis of phosphoinositides by LC/MS was carried out using a derivatization method. Although derivatization of phosphoinositides significantly increases detection in LC/MS assays (30, 36), chromatographic separation of methyl-derivatized, positional isomers is poor. For example, the bisphosphoinositide PIP2 can be PI(3,4)P2, PI(4,5)P2, or PI(3,5)P2. Each of these isomers is involved in distinct cellular signaling processes (37) and resides in specific subcellular locations, yet the methyl-derivatization method cannot distinguish between the three isomers. We thus sought to determine which of the three bisphosphoinositides is altered in NPC1 using a reversed-phase chromatographic method developed in our laboratory by modifying a previously published protocol (38). In particular, we incorporated piperidine as a mobile phase modifier to improve separation and increase ionization without derivatization of phosphoinositides in 3 and 9 week old animals. Using this approach, we observed a statistically significant decrease of PI(4,5)P2 in 9 week old Npc1−/− mice compared with control Npc1+/+ mice (Fig. 5). The molecular assignment was validated by retention time matching with commercial standards and MS/MS fragmentation matching (supplemental Fig. S17). In contrast, we did not observe signals for PI(3,4)P2 and PI(3,5)P2 compared with commercial standards, possibly because the concentrations of these bisphosphoinositides were below the limit of detection. However, when comparing the methyl-derivatized data (Fig. 3A) to the underivatized data

![Fig. 3. LC/MS analysis of methyl-derivatized selected (20:4/18:0) phosphoinositides in myelin isolated from cerebellar tissue of the NPC1 mouse model. LC/MS analysis of total (A) PIP2, (B) PIP, and (C) PI in cerebellar tissue extracts from 3 and 9 week old Npc1+/+ and Npc1−/− mice (N = 3 for each genotype and n = 3 technical injections). Significance was calculated by an unpaired t-test (**P < 0.0001 and ***P = 0.008). Standard deviation is shown by the error bars.](image)

![Fig. 4. LC/MS analysis of methyl-derivatized selected (20:4/18:0) phosphoinositides in myelin isolated from cerebellar tissue of the NPC1 mouse model. LC/MS analysis of total (A) PIP2, (B) PIP, and (C) PI in myelin isolated from cerebellar tissue of 7 week old Npc1+/+ and Npc1−/− mice (N = 3 for each genotype and n = 3 technical injections). Significance was calculated by an unpaired t-test (*P < 0.05 and **P < 0.005). Standard deviation is shown by the error bars.](image)
No differences were observed between the two genotypes. Using Western blot analysis, we probed the transfer to explain the observation of decreased phosphoinositide metabolism were altered in NPC1 mice other related enzymes and transfer proteins involved in either 3 or 9 weeks. We also investigated the possibility that in the cerebellum at \( \text{Fig. 6} \). We observed no significant decrease observed. The lower levels of PI(4,5)P2 likely impair proper sec-

dation in the cerebella in the NPC1 mouse model at 3 and 9 weeks \((N = 3 \text{ for each genotype})\). Significance was calculated by an un-

paired *test \((* P < 0.05)\). Standard deviation is shown by the error bars.

**Alterations in PI4k2α levels are observed in the Npc1-null mouse model**

The synthesis and degradation of phosphoinositides is controlled by a number of kinases and phosphatases \((39)\). Therefore, we sought to evaluate the expression of two well-documented enzymes responsible for PI(4,5)P2 synthesis in the cerebellum of 3 and 9 week old \( \text{Npc1}\text{+/+} \) and \( \text{Npc1}\text{−/−} \) mice. Western blot analysis of PI4k2α and Pip5k1α revealed significantly increased expression of PI4k2α in \( \text{Npc1}\text{−/−} \) compared with \( \text{Npc1}\text{+/+} \) mice at both the 3 and 9 week time points \((\text{Fig. 6})\). We observed no significant change in the expression of Pip5k1α in the cerebellum at either 3 or 9 weeks. We also investigated the possibility that other related enzymes and transfer proteins involved in phosphoinositide metabolism were altered in NPC1 mice to explain the observation of decreased phosphoinositides. Using Western blot analysis, we probed the transfer protein Pitpn \((\text{Fig. 6})\) and several phosphatases, including 5-phosphatase (Inpp5e), 3-phosphatase (Pten), and Sac1. No differences were observed between the two genotypes \((\text{supplemental Fig. S19})\).

**DISCUSSION**

**Alterations of phosphoinositides in NPC1 disease**

The results presented herein provide evidence for the alteration of phosphoinositide metabolism in NPC1 disease beyond PI P2. Studies linking changes in PI, PIP, and PIP2 with NPC1 have been limited. Specifically, a differential proteomic study in the cerebellum and cerebral cortex of the \( \text{Npc1}\text{-null} \) mouse model at a terminal age indicated alterations in inositol biosynthesis \((17)\). Further indirect evidence for the link between phosphoinositides and the NPC1 disease involved changes in protein kinase C (PKC) expression and signaling pathways in which PI P2 plays a central role. In particular, several reports have indicated impaired PKC signaling in NPC1 disease \((40-43)\). Vivas et al. \((16)\) reported decreased PI P2 levels in the I1061T point mutation model of NPC1 linking plasma membrane deficits to ion channel dysfunction. Additionally, it was suggested that the metabolism of PI P2 to generate secondary messengers, including calcium, may be disrupted, contributing to the recently reported calcium signaling defects in NPC1 \((44)\).

**Potential implications of alterations in PI(4,5)P2 for PI(3,4,5)P3 signaling**

Seven different forms of phosphoinositides have been reported in mammalian cells \((45)\). Each phosphoinositide can be interconverted to other phosphoinositides by meta-
bolic reactions via substrate specific kinases and phosphatases \((\text{supplemental Fig. S20})\). Among these different forms, PI(4)P and PI(4,5)P2 are the most abundant mono-
and bisphosphorylated forms, respectively, with numerous functions in membrane trafficking and cell signaling \((26, 46)\). Therefore, it is reasonable to infer that the decreases observed in these studies have major contributions from these two isomers. The 5’ position in the inositol ring of PI(4)P is phosphorylated by type I phosphatidylinositol-4-phosphate 5-kinases in the plasma membrane and Golgi apparatus as the main synthesis route of PI(4,5)P2 \((47)\). Phosphorylation of PI(4)P in the 3’ position of PI(4,5)P2 occurs via class I phosphoinositide-3-kinase to form PI(3,4,5)P3, one of the most important products of PI(4,5)P2 phosphorylation. PI(3,4,5)P3 serves as a secondary messenger that propagates signals from receptor tyrosine kinases and G protein-coupled receptors, which in turn regulate cell-cycle progression, cell death, and cytoskeleton rearrangement either directly or indirectly \((47)\). The central Akt signaling pathway relies on the generation of PI(3,4,5)P3 for activation. The lower levels of PI(4,5)P2 likely impair proper sec-

**Phosphoinositides and demyelination**

We observed decreased levels of phosphoinositides in myelin-enriched fractions from the \( \text{Npc1}\text{-null} \) mouse model. These observations suggest that the decrease observed is not cell type-specific \((\text{e.g., not limited to neurons})\) and includes changes in glial cells. Myelin basic protein, the critical protein constituent of myelin sheaths, has been shown to bind PI P2 \((50)\). This interaction was shown to be cholesterol-dependent and could be modulated by calcium.
levels (51). In line with these observations, the disease pathophysiology of NPC1, which is characterized by cholesterol storage, has a demyelination component (52) as well as a calcium defect (53). The observation that phosphoinositide levels may be altered in NPC1 disease in myelin-enriched fractions may extend to other demyelination diseases (e.g., genetic lysosomal glycosphingolipidoses such as Krabbe disease and Gaucher disease), as these have been shown to exhibit deficits in the Akt signaling pathway (54). Notably, Western blot analysis of myelin-enriched fractions did not reveal significant differences in the myelin isoform levels in control versus Npc1-null mouse cerebellar lysates (supplemental Fig. S21). This suggests that the decrease in phosphoinositides is not merely due to a decrease in myelin composition of myelin basic protein or binding but rather occurs via a less direct mechanism(s) that may include contributions of cholesterol imbalance and distribution.

**Regulatory enzymes of PI(4,5)P2**

PI(4,5)P2 is a versatile membrane-associated signaling lipid that can be synthesized from PI(4)P by kinases that act on the 5′ position of the inositol ring or from PI(5)P by kinases acting on the 4′ position. In the current studies, we observed increased expression of Pi4k2α (54 kDa) and no significant change in Pip5k1α (62 kDa) and Pitp1 (32 kDa) in the cerebellum of Npc1−/− mice. Fold change is reported as relative to Gapdh. Significance was determined using an unpaired t-test with Welch’s correction (*P < 0.05). Spliced sites of the Western blots are shown on the left side of each blot.

It is important to note that although the expression of Pi4k2α was increased in Npc1−/− mice, we observed decreased levels of total PIP2, including PI(4,5)P2, suggesting that whereas the major route of PI(4,5)P2 biosynthesis is not impaired, an alternative secondary route may be affected in the disease. Pi4k2α can be activated by the small GTPase ARF6 in a GTP- and phosphatidic acid-dependent manner (56). Interestingly, ARF proteins have also been implicated in the activation of PI4 kinases, which produce the PIP2 precursor, PI(4)P (57). Depletion of cytosolic sterols results in an increased expression of Pi4k2α. Accordingly, the enzymatic activity can be regulated by membrane cholesterol concentration (58). Therefore, the alterations in lipid and enzyme levels observed in the NPC1-null model could also play a role in the upregulation of Pi4k2α. Notably, this enzyme is not directly responsible for the loss of PIP or PIP2 levels in NPC1.

**CONCLUSION**

In summary, we have performed MALDI-MSI, LC/MS, and Western blot analysis to investigate phosphoinositide levels and distributions in the NPC1 disease. These studies revealed alterations in phosphoinositides and related enzymes in the cerebellum of Npc1−/− mice compared with controls. LC/MS analysis in whole tissue lysates confirmed decreased levels of total PIP2 in the Npc1-null mouse compared with controls, as well as alterations in the levels of the total PIP and PI. Similar findings were also observed in enriched myelin fractions. To determine which of the three structural isomers of PIP2 was contributing to the decrease observed in bisphosphoinositides, additional LC/MS assay development was carried out. These studies revealed that PI(4,5)P2 was decreased in the Npc1-null mouse.
model animals. However, we cannot exclude the possibility that other PIP2 isomers are altered, as their concentration was below the limits of detection. Last, we revealed the possibility that other PIP2 isomers are altered, as their concentration was below the limits of detection. Last, we revealed that phosphoinositide metabolism and signaling pathways are altered in the Npc1-null mouse model and may contribute to the pathophysiology of NPC1 disease.

Data availability
All data are provided within the main text and supplemental document. Raw LC/MS data are freely available at ftp://massive.ucsd.edu/MSV000083287.

The authors would like to thank Wonhwa Cho and Laura Sanchez for insightful discussions.

REFERENCES
1. De Craene, J-O., D. L. Bertazzi, S. Bär, and S. Friant. 2017. Phosphoinositides, major actors in membrane trafficking and lipid signaling pathways. Int. J. Mol. Sci. 18: 654.
2. Di Paolo, G., and P. De Camilli. 2006. Phosphoinositides in cell regulation and membrane dynamics. Nature. 443: 651–657.
3. Watt, S. A., G. Kular, I. N. Fleming, C. P. Downes, and J. M. Luccocq. 2002. Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C δ1. Biochem. J. 363: 657–666.
4. Billcliff, P. G., and M. Lowe. 2014. Inositol lipid phosphatases in membrane trafficking and human disease. Biochem. J. 461: 159–170.
5. Schulze, H., and K. Sandhoff. 2011. Lysosomal lipid storage diseases. Cold Spring Harb. Perspect. Biol. 3:a004804.
6. Fu, R., N. M. Yanjanin, S. Bianconi, W. J. Pavan, and F. D. Porter. 2010. Oxidative stress in Niemann-Pick disease, type C. J. Mol. Genet. Med. 101: 214–218.
7. Yu, W., J. S. Gong, M. Ko, W. S. Garver, K. Yanagisawa, and M. Michikawa. 2005. Altered cholesterol metabolism in Niemann-Pick type C1 mouse brains affects mitochondrial function. J. Biol. Chem. 280: 11731–11739.
8. Liao, G., Y. Yao, J. Liu, Z. Yu, S. Cheung, A. Xie, X. Liang, and X. Bi. 2007. Cholesterol accumulation is associated with lysosomal dysfunction and autophagic stress in Npc1−/− mouse brain. Am. J. Pathol. 171: 962–975.
9. Vog, C. K., and T. Kirkegaard. 2019. Animal models for Niemann-Pick type C: implications for drug discovery & development. Expert Opin. Drug Discov. 14: 499–509.
10. Loftus, S. K., J. A. Morris, J. Z. Gu, C. Cummings, A. Brown, J. Ellison, K. Ohno, M. A. Rosenfeld, D. A. Tagle, et al. 2007. Identification of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. Science. 277: 232–235.
11. Ko, D. C., L. Milenkovíc, S. M. Beier, H. Manuel, J. Buchanan, and M. P. Scott. 2005. Cell-autonomous death of cerebellar Purkinje neurons with autophagy in Niemann-Pick type C disease. PLoS Genet. 1: 81–95.
12. Fontaine, R., C. Sarraf, H. Fujisawa, B. Tortelli, J. Zhang, C. Davidson, S. U. Walkley, J. H. Bagel, C. Vite, N. M. Yanjanin, et al. 2013. Identification of Niemann-Pick C1 disease biomarkers through sphingolipid profiling. J. Lipid Res. 54: 2800–2814.
13. Pettazzoni, M., R. Froisart, C. Pagan, M. T. Vanier, S. Ruet, P. Latour, N. Guiffon, A. Fouliquet, D. P. Germain, T. Levade, et al. 2017. LC/MS/MS multiplex analysis of lysosphingolipids in plasma and amniotic fluid: a novel tool for the screening of sphingolipidoses and Niemann-Pick type C disease. PLoS One. 12: e0181700.
14. Boenzi, S., F. Deodato, R. Taurisano, D. Martinelli, D. Verrigni, R. Carrozzo, E. Bertini, A. Pastore, C. Dionisi-Vici, and D. W. Johnson. 2014. A new simple and rapid LC–ESI–MS/MS method for quantification of plasma oxysterols as dimethylaminobutyrate esters. Its successful use for the diagnosis of Niemann-Pick type C disease. Clin. Chim. Acta. 437: 93–100.
15. Besley, G. T. N., and M. Elleder. 1986. Enzyme activities and phospholipid storage patterns in brain and spleen samples from Niemann-Pick disease variants: a comparison of neuropathic and non-neuropathic forms. J. Inherit. Metab. Dis. 9: 59–71.
16. Vivas, O. A., S. A. Tiscione, R. E. Dixon, D. S. Ori, and E. J. Dickson. 2019. Niemann-Pick type C disease reveals a link between lysosomal cholesterol and PtdIns(4,5)P2 that regulates neural excitability. Cell Rep. 27: 2636–2648.e4.
34. Canonico, B., E. Cesarini, S. Salucci, F. Luchetti, E. Falcieri, G. Di Sario, F. Palma, and S. Papa. 2016. Defective autophagy, mitochondrial clearance and lipophagy in Niemann-Pick type B lymphocytes. *PLoS One.* 11:e0165780.

35. Torres, S., E. Balboa, S. Zanlungo, C. Enrich, C. Garcia-Ruiz, and J. C. Fernandez-Checa. 2017. Lyosomal and mitochondrial liaisons in Niemann-Pick disease. *Front. Physiol.* 8:982.

36. Clark, J., K. E. Anderson, V. Juvin, T. S. Smith, F. Karpe, M. J. Wakelam, L. R. Stephens, and P. T. Hawkins. 2011. Quantification of PtdIns(3)P molecular species in cells and tissues by mass spectrometry. *Nat. Methods.* 8:267–272.

37. Downes, C. P., A. Gray, and J. M. Lucocq. 2005. Probing phosphoinositide functions in signaling and membrane trafficking. *Trends Cell Biol.* 15:259–268.

38. Wenk, M. R., L. Lucast, G. Di Paolo, A. J. Romanelli, S. F. Suchy, R. L. Nussbaum, G. W. Cline, G. I. Shulman, W. McMurray, and P. De Camilli. 2003. Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat. Biotechnol.* 21:813–817.

39. Tolas, K. F., and L. C. Cantley. 1999. Pathways for phosphoinositide synthesis. *Chem. Phys. Lipids.* 98:69–77.

40. Garver, W. S., G. S. Hossain, M. M. Winscott, and R. A. Heidenreich. 1999. The Npc1 mutation causes an altered expression of caveolin-1, annexin II and protein kinases and phosphorylation of caveolin-1 and annexin II in murine livers. *Biochim. Biophys. Acta.* 1453:193–206.

41. Walter, M., F. W. Chen, F. Tamari, R. Wang, and Y. A. Ioannou. 2009. Endosomal lipid accumulation in NPC1 leads to inhibition of PKC, hypophosphorylation of vimentin and Rab9 entrapment. *Biol. Cell.* 101:141–152.

42. Tamari, F., F. W. Chen, C. Li, J. Chaudhary, and Y. A. Ioannou. 2013. PKC activation in Niemann pick C1 cells restores subcellular cholesterol transport. *PLoS One.* 8:e74169.

43. Peter, F., S. Rost, A. Rolfs, and M. J. Frech. 2017. Activation of PKC triggers rescue of NPC1 patient specific iPSC derived glial cells from gliosis. *Orphanet J. Rare Dis.* 12:145.

44. Tiscione, S. A., O. Vivas, K. S. Ginsburg, D. M. Bers, D. S. Ory, L. F. Santana, R. E. Dixon, and E. J. Dickson. 2019. Disease-associated mutations in Niemann-Pick type C1 alter ER calcium signaling and neuronal plasticity. *J. Cell Biol.* 218:4141–4156.

45. Payastrue, B., K. Missy, S. Giuriato, S. Bodin, M. Plantavid, and M-P. Gratacap. 2001. Phosphoinositides: key players in cell signalling, in time and space. *Cell. Signal.* 13:377–387.

46. Balá, T. 2013. Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol. Rev.* 93:1019–1037.

47. Zhang, P., Y. Wang, H. Sesaki, and M. Iijima. 2010. Proteomic identification of phosphatidylinositol (3,4,5) triphosphate-binding proteins in Dictyostelium discoideum. *Proc. Natl. Acad. Sci. USA.* 107:11829–11834.

48. Bi, X., J. Liu, Y. Yao, M. Baudry, and G. Lynch. 2005. Deregulation of the phosphatidylinositol-3 kinase signaling cascade is associated with neurodegeneration in Npc1−/− mouse brain. *Am. J. Pathol.* 167:1081–1092.

49. Ong, Q. R., M. L. Lim, C. C. Chua, N. S. Cheung, and B. S. Wong. 2012. Impaired insulin signaling in an animal model of Niemann-Pick Type C disease. *Biochem. Biophys. Res. Commun.* 424:482–487.

50. Musse, A. A., W. Gao, L. Homchandhuri, J. M. Boggs, and G. Harauz. 2008. Myelin basic protein as a “PI(4,5)P2-modulin”: a new biological function for a major central nervous system protein. *Biochemistry.* 47:10372–10382.

51. Weil, M. T., W. Mobius, A. Winkler, T. Ruhwedel, C. Wrzos, E. Romanelli, J. L. Bennett, L. Enz, N. Goebels, K. A. Nave, et al. 2016. Loss of myelin basic protein function triggers myelin breakdown in models of demyelinating diseases. *Cell Rep.* 16:314–322.

52. Kodachi, T., S. Matsumoto, M. Mizuguchi, H. Osaka, N. Kanai, E. Nanba, K. Ohno, and T. Yamagata. 2017. Severe demyelination in a patient with a late infantile form of Niemann-Pick disease type C. *Neuropathology.* 37:426–430.

53. Tang, Y., H. Li, and J-P. Liu. 2010. Niemann-Pick disease type C: from molecule to clinic. *Clin. Exp. Pharmacol. Pharmacol.* 37:132–140.

54. Sural-Fehr, T., H. Singh, L. Cantuti-Catelvetri, H. Zhu, M. S. Marshall, R. Rehiai, M. J. Jastrzebski, M. I. Givogi, M. M. Rascnick, and E. R. Bongarzone. 2019. Inhibition of the IGF-1-PI3K-Akt-mTORC2 pathway in lipid rafts increases neuronal vulnerability in a genetic lysosomal glycosphingolipidosis. *Dis. Model. Mech.* 12:dmm036590.

55. te Vruchte, D., A. O. Speak, K. L. Wallom, N. Al Eisa, D. A. Smith, C. J. Hendriksz, L. Simmons, R. H. Lachmann, A. Cousins, R. Hartung, et al. 2014. Relative acidic compartment volume as a lysosomal storage disorder-associated biomarker. *J. Clin. Invest.* 124:1320–1328.

56. Honda, A., M. Nogami, T. Yokozeki, M. Yamazaki, H. Nakamura, H. Watanabe, K. Kawamoto, K. Nakayama, A. J. Morris, M. A. Frohman, et al. 1999. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell.* 99:521–532.

57. Godi, A., I. Santone, P. Pertile, P. Devarajan, P. R. Stabach, J. S. Morrow, G. Di Tullio, R. Polishchuk, T. C. Petrucci, A. Lunii, et al. 1998. ADP ribosylation factor regulates spectrin binding to the Golgi complex. *Proc. Natl. Acad. Sci. USA.* 95:8607–8612.

58. Waugh, M. G., S. Minogue, D. Chotai, F. Berditchevski, and J. J. Hsuan. 2006. Lipid and peptide control of phosphatidylinositol 4-kinase IIα activity on Golgi-endosomal rafts. *J. Biol. Chem.* 281:3757–3763.