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Abnormal Gangliosides are Localized in Lipid Rafts in Sanfilippo (MPS3a) Mouse Brain

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Abstract

Allogenic stem cell transplantation can reduce lysosomal storage of heparan sulfate-derived oligosaccharides by up to 27 % in Sanfilippo MPS3a brain, but does not reduce the abnormal storage of sialolactosylceramide (GM₃) or improve neurological symptoms, suggesting that ganglioside storage is in a non-lysosomal compartment. To investigate this further we isolated the Triton X100-insoluble at 4 °C, lipid raft (LR) fraction from a sucrose-density gradient from cerebral hemispheres of a 7 month old mouse model of Sanfilippo MPS3a and age-matched control mouse brain. HPLC/MS/MS analysis revealed the expected enrichment of normal complex gangliosides, ceramides, galatosylceramides and sphingomyelin enrichment in this LR fraction. The abnormal HS-derived oligosaccharide storage material was in the Triton X100-soluble at 4 °C fractions (8–12), whereas both GM₃ and sialo [GalNAc]lactosylceramide (GM₂) were found exclusively in the LR fraction (fractions 3 and 4) and were >90 % C18:0 fatty acid, suggesting a neuronal origin. Further analysis also revealed a >threefold increase in the late-endosome marker bis (monoacylglycerol) phosphate (>70 % as C22:6/22:6-BMP) in non-LR fractions 8–12 whereas different forms of the proposed BMP precursor, phosphatidylglycerol (PG) were in both LR and non-LR fractions and were less elevated in MPS3a brain. Thus heparan sulfate-derived oligosaccharide storage is associated with abnormal lipid accumulation in both lysosomal (BMP) and non-lysosomal (GM₃ and GM₂) compartments.

Keywords

Gangliosides; Mass-spectrometry; Sanfilippo; Mouse brain; Bis(monoacylglycerol) phosphate; Lipid rafts

Introduction

The application of Mass-Spectrometry (MS) to brain lipidomics has mostly confirmed results previously obtained by laborious lipid isolation and gas-liquid chromatographic analysis. However, HPLC/MS/MS technology is clearly superior in terms of sensitivity and has enabled us to analyze minor lipids such as sphingosines, sphingosine-1-phosphates, and dihydro-forms of sphingolipids [1–6]. Recent studies have shown the applicability of HPLC/MS/MS to identify abnormal lipids in small samples of brain from lysosomal storage disease patients, and multiple sclerosis patient frozen autopsy brain [5, 7] suggesting that it could be

used to monitor therapeutic efforts. Fuller et al. [4] used a conduritol-b-epoxide model of Gaucher disease to show that glucosylceramides accumulated extralysosomally and were found in lipid rafts (LRs) by MS/MS analysis. MS/MS was also used to identify the abnormal sphingolipids in Niemann-Pick type C [8] and recent reports suggest that the disruption of lipid rafts with 2-hydroxypropyl- β -cyclodextran has therapeutic potential in Niemann-Pick type C disease [9, 10]. In addition, the ability to distinguish phosphatidylglycerol (PG) from its bioactive product BMP (bismonoacylphosphorylglyceride or bis-lyso phosphatidic acid) has opened up the potential to study the role of this endosomal structural lipid in the pathology of the lysosomal storage diseases [11]. Almost all human autopsy material is available as long-term frozen samples, precluding any subcellular fractionation attempts, but it is possible to isolate detergent-resistant membranes (DRMs or Lipid rafts (LRs)) from frozen samples which are free of lysosomal hydrolases and lysosomal storage material and analyze their lipid composition [12]. McGlynn et al. [13] used confocal microscopy and specific antibodies to identify the presence of secondary ganglioside accumulation in mouse models of several mucopolysaccharidosis (MPS) disorders (types I, IIIA, IIIB, and VII) and observed that in MPSIIIA, sialolactosylceramide (GM₃ ganglioside) only partially co-localized with the primary storage material (HS). We therefore used HPLC/MS/MS to characterize the LR and non-LR lipids, especially sphingolipids, BMP and its likely precursor PG, in SanFilippo MPSIIIA mice, in order to explain why there is such difficulty in treating brain lysosomal storage diseases where normally minor gangliosides such as GM3 and sialo [GalNAc]lactosylceramide (GM2) accumulate [13–16].

Since the coining of the term lipid rafts (LR) by Kai Simons, there has been intense use of this isolation procedure to try to answer many aspects of membrane signaling and pathology [17–25]. Based on the idea that in lysosomal storage diseases there could be perturbations in late endocytic functions leading to abnormal lipid raft composition and trafficking [17], we carried out a detailed lipidomics study of a pathological lysosomal storage disease brain. LRs were isolated from frozen mouse brain by a slight modification of the 1 % Triton X-100 extraction and sucrose-density gradient fractionation described for cells [4, 12]. The buoyant low density fractions 3 and 4 have a high content of lipids and very little protein whereas non-LR fractions 6–12 have very little sphingolipid and most of the protein. Known LR proteins such as caveolin-1, flotillin-1, contactin-1, annexin VI, GTP binding proteins and NAP-22 are also detected almost exclusively in the LR fraction whereas non-LR fractions contain Golgi markers (beta-COP), non-LR plasma membrane markers (e.g: Transferrin receptors), cytoskeletal proteins (alpha-tubulin), ER proteins (calnexin), and mitochondrial proteins (ATP synthase) [18, 19]. Detailed ESI-Mass-Spec analysis of Triton X-100 LR proteins revealed >400 species, mostly G-protein linked proteins and surprisingly very little evidence of myelin proteins such as proteolipid protein and MBP [18]. Myelin proteins can be detected in LRs but this requires more vigorous isolation procedures. We therefore used mild isolation techniques to avoid isolating significant amounts of myelin and report significant storage of abnormal sphingolipids (especially gangliosides) and unexpected distributions of bioactive lipids in LRs whereas the major lysosomal heparan sulfate storage material was in non-LR fractions.

Methods

Animal Model

The MPS3a mouse carries a naturally occurring missense D31N mutation in the catalytic site of N-sulfoglucosamine sulfohydrolase and accumulates partially degraded heparan sulfate fragments in lysosomes. Storage is predominantly in the central nervous system [13, 14]. Brain isolated from these animals at 32 weeks of age was compared to tissue from comparably aged C57BL/6 mice.

LR Isolation

Lipids from brain samples (50 mg fresh weight of mouse cerebral hemisphere) were extracted with 2 ml of 1 % TritonX-100/MES buffer-NaCl (pH 6.5) using 50 strokes of a Dounce homogenizer. Following low-speed centrifugation at 700 g to remove debris and myelin material, the supernatant was mixed with 2 ml of 1 % Triton X-100 in 80 % sucrose/MES buffer and placed in an ultracentrifugation tube. 5 ml of 30 % sucrose/MES was layered on top of this fraction, followed by 3 ml of 5 % sucrose/MES and the samples were ultracentrifuged at $39,000\times g$ for 17 h. Typically, an opalescent band was seen in Fraction 4 and this band typically contained LR protein markers such as Flotillin-1 [17]. Twelve, 1 ml fractions were removed from the top of the tube and samples of these (0.01 ml) were subjected to lipid extraction according to Folch and MS/MS analysis as described by Fuller et al. [4, 24].

Mass-Spectrometric Analysis

Samples were made up to 0.1 ml and an internal standard mix (0.024 ml) containing 400 pmol of the following non-physiological lipids was added. Ceramide (C17:0), Glucosyl-Ceramide (16:0d3), Lactosyl-Ceramide (16:0d3), phosphatidylcholine (14:0/14:0), Phosphatidylglycerol (14:0/14:0), BMP (14:0/14:0), phosphatidylinositol (14:0/14:0), GM₂ ganglioside, and cholesterol ester (17:0) [13]. Some ganglioside analyses were performed separately using GM₁d3 as internal standard. Briefly, the mixture was shaken for 10 min following addition of CHCl₃: CH₃OH (2: 1 v/v), water (0.4 ml) was added and the biphasic mixture separated and concentrated under nitrogen gas at 40 °C. Samples were reconstituted in methanol containing 20 mM ammonium formate and 0.06 ml aliquots were put into 96 well microtiter plates for analysis. Cholesterol was determined by reference to a cholesterol ester 17:0 internal standard after conversion to cholesterol ester by addition of 0.2 ml of acetylchloride:CHCl₃ (1:5 v/v).

Samples were cleaned up by HPLC to remove traces of sucrose and detergent and for BMP analysis, samples were additionally cleaned up with an Altima C18 column to remove major lipids such as PC [4, 24]. Samples were analyzed by ESI-MS/MS with a PE Sciex API 3000 triple-quadrupole mass spectrometer equipped with a turboion-spray source (200 °C) and Analyst 1.1 data acquisition system as described previously [4, 24]. Relative levels were determined by relating peak area to the internal standard. Sphingolipids (Cer, and glycosylceramides etc.) were quantified in the positive ion mode using the m/z product ion of 264 corresponding to the sphingosine base and for SM and phosphatidylcholine the m/z product of 184 corresponding to the phosphocholine head group. Other phospholipids were quantified in the negative ion mode by using the m/z product ions corresponding to the appropriate fatty acid.

Measurement of BMP and PG

A rapid, sensitive method was developed by Meickle et al. [24] to quantify individual BMP species in complex lipid mixtures containing its structural isomer phosphatidylglycerol (PG). Instead of a 30 min LC separation [24] prior to ESI-MS in negative ion mode, we used ESI-MS/MS in positive ion mode, with a short LC step to reduce signal suppression by other lipids such as PC. Precursor ion scans for the mass to charge ratio of 153.0 (corresponding to the glycerophosphate part) and m/z corresponding to individual fatty acids (e.g: 281.3 for C18:1) were used to identify individual PG/BMP species. For example, precursor ion scanning for m/z 153.0 gave a signal at m/z 775.5 which could result from either a PG or BMP 36.1 species since fragmentation of BMP can occur either side of the phosphate, leading to two different products for asymmetric BMP FA species compared to symmetrical species. Signals from the asymmetrical BMP species were therefore corrected by multiplying by a factor of 2. The PG/BMP species identified in negative ion mode were

then subjected to multiple reaction (MRM) analysis to distinguish PG/BMP species. Individual PG species were quantified by measuring the neutral loss of 189 Da, corresponding to the ammonium adduct of the glycerophosphate. Neutral loss of the ammonium adduct of the glycerophosphate cannot occur in the BMP species since both glycerol structures are acylated. Aberrant signals were minimized by adjusting the collision energy using the C14/14 standards of BMP and PG. The PG/BMP assay was linear within the range 22,000 pmol/ml. Significance of the data was assessed as described previously [4].

All results were related to the protein content of the Triton X-100 tissue extract prior to addition to the gradient in the ultracentrifuge tube and represent the average of three analyses of duplicate samples.

Results

Lipid Rafts (LR)

The LR isolation technique described gave a minor protein peak in Fraction 4 (Fig. 1a). The LR in Fraction 4 was verified by Western blot analysis using an antibody to Flotillin-1 (Fig. 1b).

GM₁-Ganglioside is in LRs

Analysis of the major ganglioside GM1 (whose structure was determined by Ledeen and Salsman in 1963) [25] revealed it to be entirely present in LRs and to be present as the expected mixture of C18:1 and C20:1 sphingosine bases acylated by C18:0 fatty acid (Fig. 2). There were no qualitative or quantitative differences in GM1 between control and MPS3a mouse brain. The relative amounts of GM1, GD1 and GT1 gangliosides were also unchanged in control and MPS3a brain (Fig. 3).

GM3 and GM2–gangliosides are present in LRs and only in MPS3a brain

The ganglioside species present in very low amounts in control mouse brain LRs (GM2 and GM3) were readily detectable in MPS3a brain (Fig. 4a, b). The LR isolation profile showed >95 % of GM3 to be in Fractions 3 and 4, with essentially no GM3 in non-LR fractions. Of the species of GM3 detected, (16:0, 18:0, 20:0, 22:0, 24:0 and 24:1), C18:0 was >90 %, (Fig. 4b) suggesting an origin from brain gangliosides. GM2 was quantified with a d3-GM1 internal standard and showed a similar increase in LRs from MPS3a cortex (Fig. 5). The normally undetectable GM3 precursor Lactosylceramide was detected as dihexosylceramide in MPS3a brain LRs but the quantification is unreliable at present.

Ceramides

Quantification of C18:1 sphingosine acylated by 16:0, 18:0, 20:0, 22:0, 24:0 and 24:1 (which constitutes >80 % of mouse brain ceramides) revealed no significant differences between control and MPS3a brain and >95 % of ceramides were in LRs including the major species C24:1 (Fig. 6a).

Glycosyl-Ceramides

Analysis of non-hydroxylated fatty acid-containing species (presumably Galactosylceramides) revealed >95 % of C18: 1 sphingosine acylated by 16:0, 18:0, 20:0, 22:0, 24:0 and 24:1. C24:1 to be in LRs. The C24:1 GlyCer (most likely galactosylceramide but indistinguishable from glucosylceramide by this technique) was the major species and none showed any differences between controls and MPS3a mice.

Sphingomyelins and Cholesterol

Although C18:1/C18:0 SM was 75 % of the total SM pool it was >95 % in LR and there was no difference between control and MPS3a brains. The 16:0, 16:1, and 18:1 species were also >95 % in Rafts (Fig. 6c) but the longer chain 22:0 (Fig. 6d), and 24:0 forms were only 20 % in LR. Overall these longer chain forms showed a 25 % increase in MPS3a cerebral cortex but they only represent 5 % of total SM so the significance of this is unknown. Cholesterol was distributed between LR (28 %) and non-LR fractions, but reduced to 20 % in LR in MPS3a. In many lipid storage diseases there is increased cholesterol, the exception being GM2-gangliosidosis [17], so the role of increased or decreased LR cholesterol in pathology deserves further study.

Oligosaccharide Storage Material

The major lysosomal storage material in MPS3a brain, as reported previously [26], was heparan sulfate disaccharide (GlcNS-UA) (80 %) and heparan sulfate tetrasaccharide (17 %). Both were essentially absent from normal brain. There was no evidence for disaccharide in LR and only traces of tetrasaccharide in LR, suggesting that the heparan sulfate-derived storage material does not associate with the elevated gangliosides in LR but does associate with the BMP in non-LRs. (Fig. 7).

Bis-Monoglycerolphosphate (BMP/lyso-BPA)

In contrast to its precursor PG, BMP was mainly (>95 %) in non-LRs. C22:6/22:6 BMP constituted 75 % of BMP species (Fig. 8a) with lesser amounts of 20:4/22:6, 18:1/22:6, 18:1/22:4 and 18:1/18:1, which are potential intermediates in its synthesis. All BMP species showed a >twofold elevation in cerebral hemispheres from MPS3a mice compared to control littermates (Fig. 8), as predicted for a lysosomal storage disease [27].

Phosphatidylglycerol

The lack of correlation between PG and BMP in terms of presence in LR and fatty acid composition was especially striking since PG is the biosynthetic precursor of BMP [28]. About 25 % of mouse brain PG was in LR and in contrast, less than 5 % of BMP was in LR. PG 32:0, (about 10 % of all PG) was essentially 100 % in LR. In mouse brain, the predominant PG (34:1) represented 65 % of total PG and is most likely C16:0/C18:1. Of this, 40 % was in LR (Fig. 8g). In contrast, the polyunsaturated forms (36:2, 36:3 and 38:5), which could be intermediates in the 4-step synthesis of BMP from PG [28], were predominantly in non-LR fractions. (Fig. 8h). This transit of PG from plasma membrane (LR) to BMP in endosomes/lysosomes has not previously been demonstrated.

Other Lipids

Using the phosphorylcholine internal standard for phosphatidylcholine we recorded only the total number of carbon atoms and double bonds of the two fatty acids. C32 was the major species and together with C32:1 was >95 % in Rafts. C34:1 was almost equal to C32 but was only 80 % in Rafts. The polyunsaturated species, C34:2, 36:2, 36:4 and 38:4 were present in LR but were predominantly in non-Rafts. Levels in MPS3a were similar to controls. Most species of Phosphatidylinositol were 20 % in LR with C18:0/16:0 and 18:0 closer to 30 %, as expected for saturated species. C18/20:4 was the major species but no significant differences were seen between control and MPS3a brain. Phosphatidylserine and phosphatidylethanolamine were heterogeneous and distributed between LR and non-LR without showing any clear pathological correlation.

Discussion

We present evidence that in MPS3a brain, GM3 and GM2 gangliosides accumulate in the LR compartment rather than in the lysosome (where HS accumulates), which might explain why they are less accessible to therapeutic attempts [4, 10, 15, 16]. Although there is still considerable controversy as to whether or not LR are biological/physiological entities as opposed to biophysical entities, the study of the movement of biologically active molecules in and out of LRs has given some important insights into function [17–23]. The concept of LRs has helped understand how GPI-anchored proteins might function and how modification of proteins by hydrophobic palmitoylation, myristoylation and prenylation may facilitate their targeting to specific molecules in membranes. In addition, certain transmembrane proteins (for example, neutral sphingomyelinase) can be shown to be recruited into LRs as part of their activation, a mechanism most likely involving palmitoylation) [29, 30]. The concept of LRs has also shed some light on the reason for fatty acid heterogeneity of many membrane lipids and in this study we reported abnormal amounts of several forms of BMP, all in the same fraction as the stored HS, whereas the precursor PG was in both LRs and the HS fraction. We also report that some forms of SM do not associate primarily with LRs but in agreement with previous studies [4], most sphingolipids are in LRs. It has been possible to detect the depolarization-induced enhancement of the cholesterol-dependent association of syntaxin with Brij-98 synaptosomal LRs but not with LubrolWX LRs, under the same conditions in which prolonged neurotransmitter release from synaptosomes is stimulated, suggesting a physiological relevance to the study of brain LRs. Similarly the observation that segregation of the p75 neurotrophin receptor and the protease involved in its shedding (TACE) can be achieved by their differential localization into LRs [18–22]. Further, AMPA receptors have recently been shown to localize within specific regions of synaptic membranes rich in GM1 ganglioside [31] and the abnormal GM3 and GM2 are likely playing a role in brain pathology of MPS3a.

Detailed analysis of lipid changes in biological samples has recently become possible because of the development of tandem MS technology [1–6] and we have applied this technology to the study of a range of lipids in LRs from a mouse model of MPS3a. In this disease, the primary storage material is non-Lipid (heparan sulfate (HS)-derived oligosaccharides) but immunocytochemical studies [15] indicate that abnormal GM3 is a more-refractory part of the pathology in MPS3a and therefore a challenge for therapeutic reversal. In studies by Lau et al. [15] virtually no GM3 immunoreactivity was observed in the amygdala and inferior colliculi of wild-type mice whereas substantial GM3 staining was detected in untreated 19–29 week-old MPS IIIA mice in these regions. This GM3 storage was not reduced by up to 25 weeks post-allogeneic stem cell transplantation of the MPS IIIA mice [15]. In contrast, the transplantation reduced heparan sulfate storage in brain by 27 % although it did not improve the condition of the mice. This suggests that irreversible GM3 ganglioside storage has pathological consequences. Using MS/MS we confirmed the accumulation of GM3 and also find elevated GM2 in LRs, whereas the major gangliosides [25] are present in normal amounts. Thus in addition to reducing lysosomal HS storage there is a need to target gangliosides in LRs as part of any therapeutic endeavor. For example, a similar storage of GM3 and GM2 gangliosides has been reported in Niemann-Pick type C (NPC) in which there is good evidence for increased cholesterol in LRs [8–10]. A combination clinical trial using N-butyldeoxynojirimycin (to reduce glycolipid synthesis) and allopregnanolone, noted an equally increased lifespan for NPC1^{−/−} mice receiving only 2-hydroxypropyl- β -cyclodextrin [10], a detergent known to bind cholesterol and disrupt lipid rafts. In contrast, the 2-hydroxypropyl- β -cyclodextrin had no clinical effect on mice with either MPS3a or GM1-gangliosidosis. This may be because the cholesterol content of LRs was reduced rather than being elevated as on NPC1^{−/−} mice.

An explanation for the accumulated gangliosides in LRs may be that proteins and lipids destined for lysosomal degradation are incorporated into multivesicular BMP-rich endosomes [32, 33] which then either fuse with lysosomes for digestion or fuse with the plasma membrane to be released as exosomes [34]. Analysis of the exosomes from brain-derived Oli-neu cells [34] revealed a composition remarkably similar to LRs, namely enrichment in cholesterol, sphingolipids such as gangliosides and saturated phosphoglycerides such as PC. Formation of these exosomes, is dependent upon ceramide (released from SM by neutral sphingomyelinase), a lipid which has been primarily implicated in the amplification of cell death signals. We find that all of the ceramides and most of the SM to be present in LRs. The exception was that 50 % of the C22 and C24 forms of SM were in non-LR fractions. The C16 and C18 forms, which have been implicated in stress-related SM hydrolysis and generation of ceramides, were all in LR fractions. The abnormal GM3 and GM2 are therefore a marker for abnormal LRs and potentially can be corrected by reversible LR disruption with Raft-modifying agents such as beta-methylcyclodextran, as being tested in human NPC therapy.

BMP has long been recognized as accumulating in lysosomal storage diseases [27] and its presumed precursor Phosphatidylglycerol (PG) [28] has a major C16/16 form which was 50 % elevated in MPS3a and which we found predominantly in LRs. This is converted to lyso-PG and then transacylated to an intermediate (38:5 or C18:1/20:4) which appears in LRs but is mostly present in elevated amounts in MPS3a non-LR fractions. A second round of PLA1 phospholipase and transacylation [28] then generates BMP (not in LRs) which is mainly C20:4/C20:4 and 22:6/22:6 in both normal and MPS3a mouse brain. Although it is not clear what role the elevated levels of BMP play in the pathology of lysosomal storage diseases, it seems likely that the elevated BMP (critical for endosome structure and function) is associated with the increased gangliosides. Since gangliosides have critical roles in repair and pathology [35] we would predict that there would be no reversal of disease pathogenesis until BMP was normalized. The transport of PG from LRs to the BMP in endosomes must be tightly regulated and of major biological significance. Since both Rafts and exosomes have been suggested to form platforms for cell signaling [34, 36], their formation by living cells further suggests that LRs do have a real biological counterpart and that LR modification needs to become a drug target in therapy of lysosomal storage diseases.

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Fig. 1. Lipid raft isolation. **a** Lipid raft isolation showing protein distribution on the sucrose density gradient from a representative MPS3a brain Triton X-100 extraction. **b** Representative Western blot of mouse brain lipid raft preparation as shown above and isolated as described in the text. Each fraction was assayed for marker Flotillin-1 content and quantification showed that >95 % of Flotillin is in Fraction #4 (the Lipid Raft fraction)

Fig. 2. HPLC/MS/MS analysis of GM1 molecular species in Control versus MPS3a brain extracted with Triton X-100 and fractionated on a sucrose density gradient as described in the text. **a** GM1 containing C_{18:1} sphingosine and C_{18:0} fatty acid is predominantly in Fraction #4 (lipid raft) in both Control and MPS3a brain. **b** GM1 containing C_{20:1} sphingosine and C_{18:0} fatty acid is predominantly in Fraction #4 (lipid raft) in both Control and MPS3a brain. Data shown is average of analyses of cerebral cortex from two different mice

Fig. 3. HPLC/MS/MS analysis of G_{M1} , G_{D1} and G_{T1} molecular species in the lipid raft Fraction (#4) in Control versus MPS3a brain, extracted with Triton X-100 and fractionated on a sucrose density gradient as described in the text. >95 % of the G_{M1} , G_{D1} and G_{T1} were in Fraction #4. The technique does not distinguish between individual disialogangliosides, G_{D1a} and G_{D1b} etc. or trisialogangliosides. No significant differences were observed. Data shown is average of analyses of cerebral cortex from two different control and MPS3a mice

Fig. 4. HPLC/MS/MS analysis of G_{M3} molecular species in Control versus MPS3a brain extracted with Triton X-100 and fractionated on a sucrose density gradient as described in the text. **a** Most of the G_{M3} in MPS3a brain is in lipid rafts (Fraction #4) with only trace amounts of G_{M3} in controls. **b** Molecular species of G_{M3} in Fraction #4, showing the relative amounts of $C_{18:1}$ sphingosine acylated by $C_{16:0}$, $C_{18:0}$, $C_{22:0}$ and $C_{24:0}$ fatty acids. Data shown is average of analyses of cerebral cortex from two different control and MPS3a mice

Fig. 5. Molecular species of GM₂ in Fraction #4, showing the relative amounts of C_{18:1} sphingosine acylated by C_{16:0} and C_{18:0}, fatty acids and C_{20:1} sphingosine acylated by C_{18:0} fatty acid (20/18) and its accumulation in MPS3a brain. Data shown is average of analyses of cerebral cortex from two different control (*open bar*) and MPS3a (*filled bar*) mice

Fig. 6.

Most sphingolipids are found in Lipid Rafts (LRs). HPLC/MS/MS analysis of the major sphingolipid molecular species in Control (*open bar*) versus MPS3a cerebral cortex (*filled bar*) extracted with Triton X-100 and fractionated on a sucrose density gradient as described in the text. Results are shown for all 12 fractions with the lipid rafts being mainly in Fr #4. **a** C_{18:1}/C_{24:1}-ceramide. **b** C_{18:1}/C_{24:1} Glyceramide. **c** C_{18:1}/C_{18:0}-sphingomyelin **d** C_{18:1}/C_{22:0}-a minor (<5 %) sphingomyelin showing presence of 85 % in non-lipid raft fractions of the sucrose-density gradient. Data shown is average of analyses of cerebral cortex from two different control and MPS3a mice

Fig. 7. HPLC/MS/MS analysis of the two major heparan sulfate-derived storage oligosaccharides in Control versus MPS3a brain extracted with Triton X-100 and fractionated on a sucrose density gradient as described in the text. Both oligosaccharides are completely absent from control brain. **a** GlcNS-UA) Disaccharide. Data shown is average of analyses of cerebral cortex from control (*open bar*) and MPS3a (*filled bar*) mice. **b** [GlcNS-UA]₂ Tetrasaccharide

Fig. 8. HPLC/MS/MS analysis of major molecular species of BMP (lyso-bis-phosphatidic acid, **a–e**) and its precursor PG (phosphatidylglycerol) in Control versus MPS3a brain extracted with Triton X-100 and fractionated on a sucrose density gradient as described in the text. Data shown is average of analyses of cerebral cortex from two different control (*open bar*) and MPS3a (*filled bar*) mice. **a** BMP containing C_{22:6}/C_{22:6} fatty acids is increased in MPS3a brain but mostly absent from Lipid Rafts. **b** BMP containing C_{20:4}/C_{22:6} fatty acids is increased in MPS3a brain but mostly absent from Lipid Rafts. **c** BMP containing C_{18:1}/C_{22:6} fatty acids is increased in MPS3a brain but mostly absent from Lipid Rafts. **d** BMP

containing C_{18:1}/C_{20:4} fatty acids is increased in MPS3a brain but mostly absent from Lipid Rafts. **e** BMP containing C_{18:1}/C_{18:1} fatty acids is increased in MPS3a brain but mostly absent from Lipid Rafts. **f** PG containing C_{16:0}/C_{16:0} fatty acids (the proposed precursor of BMP) is not increased in MPS3a brain and is mainly present in Lipid Rafts. **g** PG containing C_{18:1}/C_{16:0} fatty acids (34:1)(the proposed precursor of BMP) is not increased in MPS3a brain and is mainly present in Lipid Rafts. **h** PG containing C_{18:1}/C_{20:4} fatty acids (38:5)(the proposed precursor of BMP) is increased in MPS3a brain and is mainly present in non-Lipid Raft fractions