SPHINGOLIPIDS AND NEURONAL DEGENERATION IN LYSOSOMAL STORAGE DISORDERS.

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Complete List of Authors:	Grassi, Sara; University of Milan, Department of Medical Biotechnology and Translational Medicine Chiricozzi, Elena; Universita degli Studi di Milano, Department of Medical Biotechnology and Translational Medicine; Mauri, Laura; University of Milan, Department of Medical Biotechnology and Translational Medicine SONNINO, SANDRO; Universita degli Studi di Milano, Department of Medical Biotechnology and Translational Medicine Prinetti, Alessandro; University of Milan, Department of Medical Biotechnology and Translational Medicine
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SPHINGOLIPIDS AND NEURONAL DEGENERATION IN LYSOSOMAL STORAGE DISORDERS.

Sara Grassi, Elena Chiricozzi, Laura Mauri, Sandro Sonnino and Alessandro Prinetti

Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy

Correspondence to:

Alessandro Prinetti, Department of Medical Biotechnology and Translational Medicine, Via Fratelli Cervi 93, 20090 Segrate (Milano), Italy. Phone: +390250330376; fax, +390250330365; e-mail: alessandro.prinetti@unimi.it.

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Keywords: sphingolipids, gangliosides, sphingomyelin, sphingolipidoses, lysosome, membrane fusion

Abbreviations: Ganglioside and glycosphingolipid nomenclature is in accordance with the IUPAC-IUBMB recommendations (Chester 1998).

ASM, acid sphingomyelinase; CERT, ceramide transfer protein; ER, endoplasmic reticulum; FAPP2, four-phosphate-adaptor protein 2; LSDs, lysosomal storage disorders; LVs, intra-endolysosomal vesicles; MAMs, mitochondria-associated ER membranes; MCSs, Membrane Contact Sites; ORMDL, orosomucoid-like proteins; PM, plasma membrane; SL, sphingolipids; SM, sphingomyelin; SMase, sphingomyelinase; TFEB, transcription factor EB.

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ABSTRACT

Ceramide, sphingomyelin, and glycosphingolipids (both neutral and acidic) are characterized by the presence in the lipid moiety of an aliphatic base known as sphingosine. Altogether, they are sphingolipids particularly abundant in neuronal plasma membranes, where, via interactions with the other membrane lipids and membrane proteins, they play a specific role in modulating the cell signalling processes. The metabolic pathways determining the plasma membrane sphingolipid composition are thus the key point for functional changes of the cell properties. Unnatural changes of the neuronal properties are observed in sphingolipidoses, lysosomal storage diseases occurring when a lysosomal sphingolipid hydrolase is not working, leading to the accumulation of the substrate and to its distribution to all the cell membranes interacting with lysosomes. Moreover, secondary accumulation of sphingolipids is a common trait of other lysosomal storage diseases.

In this review article, we report on the sphingolipid chemical and biochemical properties and discuss some hypotheses about the possible links between sphingolipid incorrect catabolism and neurodegeneration.

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6 INTRODUCTION
7 Sphingolipids are amphiphilic membrane lipids characterized by the presence of
8 sphingosine, an amino-and-hydroxy alkylic long chain. After more than a century of
9 studies, we now know their structure, their physico-chemical properties, their distribution
10 and content in cells and tissues of many animal species, including humans (Sonnino *et al.*
11 2006). The final cellular site for sphingolipids is the plasma membrane, however their
12 complex metabolic pathway requires many intracellular processes, so that a not negligible
13 portion of them is also found intracellularly (Schulze *et al.* 2009, Kolter & Sandhoff 2010,
14 Sandhoff & Kolter 2003, Hannun & Obeid 2018). As amphiphilic compounds they contain a
15 hydrophilic head group, for example a saccharide, an oligosaccharide, a phosphocholine,
16 protruding into the cell aqueous environment, and a hydrophobic chain called ceramide, in
17 which the sphingosine is linked with a fatty acid via an amidic bond, inserted into the outer
18 layer of plasma membrane.
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21 Sphingolipids are typically asymmetric components of the plasma membranes enriched in
22 the outer leaflet (van Meer 2011, van Meer & Hoetzl 2010, Fujimoto & Parmryd 2016),
23 however it cannot be excluded that a minor pool of them could be transiently inserted into
24 the inner layer with the hydrophilic group facing the cytosol. The hydrophilic group, thanks
25 to the presence of carbohydrates and/or ionic charges, attracts water and ions, forms
26 hydrogen bonds and easily interacts with the group of membrane components protruding
27 from the membrane itself. At the water-lipid interface, the sphingolipid ceramide amide
28 linkage accepts hydrogen bonds on the carbonyl group and donates hydrogen bonds via
29 the N-H group, allowing the formation of a double linkage, which works as a padlock within
30 molecules. The lipid moiety inserted into the lipid layer interacts with the lipid portion of the
31 cholesterol in the membrane and with dipalmitoylphosphocholine, leading to the formation
32 of membrane domains with reduced fluidity, now worldwide known as lipid rafts (Sonnino
33 et al. 2006, Sonnino & Prinetti 2013, Sonnino *et al.* 2007). Most of the available
34 information on the composition and organization of lipid rafts relies on the preparation and
35 analysis of low-density, detergent-resistant membrane (DRM) fractions from cells, tissues
36 or even entire organisms. The notion that lipid-driven segregation of membrane
37 components could be a major mechanism for the creation of lateral order in biological
38 membranes was supported by at least a decade of studies on model membranes, before
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the formulation of the "lipid raft hypothesis" (van Meer *et al.* 1987). However, the method published by Brown and Rose describing the isolation of a putative lipid raft fraction based on its resistance to solubilization by Triton X-100 under well defined experimental conditions (Brown & Rose 1992) has hallmarked an explosion of investigations using this method to study the biological roles of lipid rafts. DRM are highly enriched in sphingolipids and cholesterol, and relatively void of bulky membrane glycerolipids (for a detailed analysis of the lipid composition of DRM prepared from primary cultured neurons, see (Prinetti *et al.* 2001)). The method, despite its enormous popularity, has elicited as well fierce opposition, based on some pieces of evidence indicating that the fraction prepared with this method might be artifactual and not reflecting the real lipid raft structure as it occurs in living cells (for a detailed discussion about this issue, see our reviews (Sonnino & Prinetti 2008, Sonnino & Prinetti 2013)). The evolution of different technologies enabling to probe the heterogeneous organization of cellular membranes suggested that "membrane domains" (among those, lipid-driven domains or "lipid rafts") are a multiple, heterogeneous, highly dynamic entities, part of a complex organization hierarchy (Bernardino de la Serna *et al.* 2016, Lu & Fairn 2018, Sezgin *et al.* 2017, Santos & Preta 2018). Isolated DRM fractions capture a non-dynamic situation representing the average or sum of different situations. In this sense, DRM are indeed an artifact (as almost any kind of biochemical preparation, defining an experimental item that is not reflecting the complex reality of the *in vivo* counterpart). On the other hand, a few diverse techniques enabling to observe organized domains in intact cell membranes (immune-electron microscopy under conditions respecting the lipid integrity of the membrane (Fujita *et al.* 2007), stimulated emission depletion nanoscopy (Iwabuchi *et al.* 2012, Eggeling *et al.* 2009) and fluorescence correlation spectroscopy (Sezgin *et al.* 2012)) have demonstrated the clustering and confinement of different glycosphingolipids at the plasma membrane level.

Sphingolipids are ubiquitous components of mammal cell membranes, which are particularly abundant in the nervous system, and, within the nervous system, in neurons (Hansson *et al.* 1977, Schnaar 2016). Thus, sphingolipids are relatively abundant components of the outer layer of neuronal plasma membranes making their concentration very high in neuronal lipid rafts (Prinetti et al. 2001). Remarkably, membrane segregation of sphingolipids in neurons seems to be higher than in any other cell type investigated so far. Lipid rafts contain a few proteins, involved in cell signaling processes, whose functional features are modulated through direct specific glycosphingolipid-protein

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interactions or through a sphingolipid-dependent dynamic membrane organization (Prinetti *et al.* 2009). As a matter of fact, several experimental approaches, which eventually lead to alterations in the organization of the plasma membrane due to changes in sphingolipid content or pattern, have been proven to be very effective in modulating cell functions.

METABOLISM OF SPHINGOLIPIDS

The sphingolipid metabolism combines several metabolic pathways, correlated with each other and aimed at obtaining and maintaining a defined cell- and stage-specific cellular content and plasma membrane pattern (Sandhoff & Kolter 2003, Yu *et al.* 2009, Furukawa *et al.* 2014). Figure 2 shows a schematic representation of the complex sphingolipid metabolic pathway.

Differentiated neurons display the more complex sphingolipid pattern within all mammalian cells (Svennerholm *et al.* 1994). In these neurons, sphingomyelin is the most abundant sphingolipid. Gangliosides are distributed, all in similar quantity, among mono-, di-, tri- and tetra-sialo compounds of the ganglio-4, Gg_4, series. The sphingolipid pattern changes during the differentiation of the nervous system and parallels the expression of the sphingolipid synthases (Yu et al. 2009, Prinetti et al. 2001, Riboni *et al.* 1990). Sphingolipids are present in low amounts in the first stages of differentiation, then their levels increase progressively as the differentiation process proceeds, before reaching a phase of the process where they remain constant for many years. This phase is then followed by a decrease in the sphingolipid content in very aged people. During the stages of neural tube formation and neural stem cells proliferation, the ganglioside pattern is characterized by the mono- and disialogangliosides of the Lac series, GM3 and GD3. Then, these gangliosides become very scarce, giving way to the more complex gangliosides of the Gg_4 series (GM1, GD1a, GT1b and GQ1b) as neurogenesis and astrocytogenesis proceed. These gangliosides reach their highest quantity with synaptogenesis and axonal/dendritic arborization (Schnaar 2016). At the onset of terminal differentiation, synthesis of galactosylceramide and sulfatide in the brain is switched on, then remains constant in mature oligodendrocytes (Norton & Poduslo 1973). Sphingomyelin is also very scarce at the beginning of differentiation growing then hyperbolically and reaching the maximum level in fully differentiated cells. In differentiated rat cerebellar granule neurons, sphingomyelin content is 1.00 nmol/10^6 cells, while gangliosides account for 0.79 nmol/10^6 cells, with a total glycerophospholipid content of 32.84 nmol/10^6 cells (Prinetti et al. 2001).

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The sphingolipid content is strictly related to the synthesis and content of sphinganine, which displays a C18/C20 sphinganine ratio progressively reduced along differentiation. The C20 species can hardly be detected in the first stages of differentiation. This is due to a different activity of the palmitoyl- and stearoylCoA acyl–serine transferases along neuronal differentiation (Chigorno *et al.* 1997), which results in the slow but continuous increase in the content of gangliosides that contain C20-sphingosine during neuronal maturation and aging (Valsecchi *et al.* 1993, Sonnino & Chigorno 2000).

De novo biosynthesis of sphingolipids starts with the synthesis of ceramide in the endoplasmic reticulum and proceeds in the Golgi apparatus (see reference (Hannun & Obeid 2018) for details). Neobiosynthesized sphingolipids reach the plasma membranes *via* vesicular transport, becoming thus components of the external leaflet (Sandhoff & Kolter 2003, van Meer & Hoetzl 2010).

Ceramide follows two paths to reach the Golgi, *via* the soluble transporter protein CERT to become sphingomyelin in the luminal site, and *via* vesicle transport to become glucosylceramide by glucosyltransferase on the cytosolic side of the membrane (van Meer & Lisman 2002). Glucosylceramide can be translocated to the luminal side of the Golgi membrane becoming substrate for the lactosylceramide synthases (D'Angelo *et al.* 2007). A part of glucosylceramide is directly transported by a specific protein from the membrane cytosolic layer of Golgi to the plasma membrane inner layer and to the outer by a flippase (Warnock *et al.* 1994).

On the other hand, the biosynthetic activity at Golgi level is not the only event influencing the final sphingolipid pattern associated with the plasma membrane. Indeed, the plasma membrane itself is an active site for metabolic remodeling of the local sphingolipids. Both hydrolytic and biosynthetic sphingolipid enzymes, associated to the plasma membranes, have *cis* and *trans* activities (Preti *et al.* 1980, Saito *et al.* 1995, Aureli *et al.* 2014a, Aureli *et al.* 2014b, Aureli *et al.* 2012, Aureli *et al.* 2011, Sonnino *et al.* 2010). Plasma membrane-associated sphingomyelinases, sialidases, hexosamindase, galactosidase, glucosidases, sphingomyelin synthase, sialyltransferase have been characterized and studied (see references (Schnaar 2016, Hannun & Obeid 2018) for details). Hydrolysis of SM by PM-associated acid or neutral SMases, and synthesis of SM from ceramide by SMS2 likely play an important role in regulating the formation, at the plasma membrane, of ceramide-enriched domains acting as signaling platforms (Zhang *et al.* 2009) or initiating vesiculation

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processes (Holopainen *et al.* 2000). Regulation of PM ceramide levels, or more complex modulation of glycosphingolipid patterns and glycosphingolipid-dependent signaling can also be achieved through the action of PM-associated glycohydrolases or glycosyltransferases (Sonnino et al. 2010). Hydrolase activities are likely modulated by the activity of proton pumps able to determine local acidification in proximity of the lipid raft microenvironment, to obtain optimum acidic pH required for the enzyme activity (Aureli et al. 2012).

Glycosylation/deglycosylation is not the only opportunity for the local modulation of PM glycosphingolipid composition. The local increase in proton concentration in proximity of glycosphingolipid-enriched membrane domains determined by the activity of the proton pumps/antiporters mentioned above favors the formation of ganglioside lactones, in particular GD1b lactones. The lactonization process deeply influences the conformational, aggregational and biological properties of gangliosides (Acquotti *et al.* 1987, Bassi *et al.* 1991), even if its biological relevance is still poorly understood. However, in human brain the content of GD1b-lactone, that is very small in young subjects, significantly increases with the aging process (Riboni *et al.* 1986).

A further opportunity for the remodeling of the PM sphingolipid pattern is represented by the release of sphingolipids or sphingolipid-rich vesicles from the plasma membrane into the extracellular environment (at least, in cultured cells (Chigorno *et al.* 2006)). On the other hand, sphingolipids present in the extracellular milieu are taken up, at least partially, by cells (not necessarily by the same cells from which they have been released), remaining associated with the cell membranes.

While the enzymology of sphingolipid *de novo* biosynthesis and remodeling at the PM level has been unveiled in many of its details, still relatively little is known about its regulation, even if the regulation of the biosynthetic pathway has been regarded for a long time as the main mechanism responsible for the formation of a cell- and stage-specific sphingolipid pattern.

Sphingolipid synthesis is mainly regulated at the transcriptional level through the control of the expression levels of ceramide synthases, sphingomyelin synthases, glycosyltransferases or transporter proteins. Indeed, the changes in cellular glycosphingolipid patterns occurring in neurons along neurogenesis, axonal arborisation and synaptogenesis, as well as in oligodendrocytes along differentiation and myelin formation, are nicely paralleled by changes in the expression of the corresponding

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glycosyltransferases (Ngamukote *et al.* 2007, Yu et al. 2009), and apparently histone acetylation of the glycosyltransferase genes represent an important regulatory in brain (Suzuki *et al.* 2011, Itokazu *et al.* 2017). An important regulation mechanism for the reaction catalyzed by the serine-palmitoylCoA transferase, which likely represents the rate-limiting step in the biosynthetic pathway, is represented by an inhibitory feedback controlled in mammals by ORMDL proteins (Gupta *et al.* 2015, Siow & Wattenberg 2012). In addition, it has been recently suggested that ceramide synthase activity could be relevantly regulated at the protein level by phosphorylation (Wegner *et al.* 2016).

However, the possibility that differential intracellular flows of different sphingolipids could influence the resulting sphingolipid patterns (independently from the expression levels of synthases) should not be neglected. In other words, the regulation of intracellular sphingolipid traffic might be of equal importance as the control of synthetic enzymes expression in determining the final sphingolipid composition of the plasma membrane. From this point of view, it is worth to recall that the mechanism of glucosylceramide delivery to the ganglioside biosynthetic machinery in the Golgi is likely not mediated by the transport protein FAPP2. It is generally assumed to be due to a vesicular transport, however this crucial point in the biosynthetic pathway of gangliosides remains elusive.

The degradation of plasma membrane sphingolipids takes place in the lysosomes, that are reached by the endocytic vesicular flow through the early and late endosomal compartment (Kolter & Sandhoff 2005). Along their route to the lysosomes, the plasma membrane sphingolipids can be diverted to the Golgi apparatus where they undergo direct glycosylation with the formation of more complex products, able in turn to reach again the plasma membrane. It has been suggested that this process might be quantitatively relevant at least for certain cell types, including neurons (Riboni *et al.* 1994), thus representing potential mechanism for the regulation of plasma membrane sphingolipid pattern at the level of intracellular traffic.

After reaching reached the lysosomes, sphingolipids are catabolized to simple compounds (Kolter & Sandhoff 2005, Kolter & Sandhoff 2010), but some glycolipid intermediates escape from the lysosomes and can reach directly the Golgi entering into the biosynthetic pathway. This salvage pathways, related to gangliosides, in neurons are not negligible from the quantitative point of view (Riboni *et al.* 1996), but very little is known about the mechanisms of escape from the lysosome and the transfer of these intermediates to the Golgi or other cellular districts, nor on the regulation of these processes. The presence of

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soluble ganglioside-protein complexes in the cytosol might reflect, at least in part, the intracellular traffic linked to the recycle of these intermediates (Sonnino *et al.* 1979). On the other hand, it is well known that lysosomes interact with many cellular organelles and with the plasma membrane. Processes of membrane fusions could be responsible for the shift of glycolipids from one cellular compartment to another (for details, see the following section).

The final degradation products, sphingosine, fatty acids and sugars, leave the lysosomes through specific membrane proteins and, in part, are recycled for the biosynthesis of new sphingolipids (Sonderfeld *et al.* 1985, Tettamanti 2004, Riboni et al. 1996). In particular, the recycling of sphingosine is very high and in some cells covers up to 80% the need of the long chain base for sphingolipid biosynthesis (Riboni *et al.* 1999). However, information on the intracellular trafficking of sphingosine, from lysosomes to the reticulum is not available. Lysosome-originated sialic acid is also recycled at a great extent for the biosynthesis of sialoglycoconjugates. Sialic acid escape from the lysosomes requires the membrane protein sialin (Prolo *et al.* 2009). The lack of sialin leads to lysosomal impairment and neurodegeneration in the pathology known as Salla's disease.
Sphingolipid lysosomal catabolism is not a simple process (Kolter & Sandhoff 2006, Kolter & Sandhoff 2010). Sphingolipids are components of the external layer of plasma membranes and upon membrane endocytosis they become components of the negatively curved luminal layer of the limiting lysosomal membrane, which is covered by a thick and complex glycocalix (Eskelinen *et al.* 2003). Thus, their accessibility to the lysosomal hydrolytic enzymes and activator proteins would be limited, resulting in a slow and difficult hydrolytic process. Small endo-lysosomal vesicles characterized by a high content of bis(monoacylglycero)phosphate, with the sphingolipids in the outer layer facing the lysosol with a topology more suitable to the enzymatic hydrolysis, are rapidly formed in the late lysosomes (Schwarzmann *et al.* 2015). Thus, both lysosomal membrane sphingolipids and endo-lysosomal vesicle membrane sphingolipids can be found in the lysosomes. The implications of lysosome fusion with the PM will be discussed in the next section.

According to this scenario, it is clear that the sphingolipid plasma membrane composition can be easily modified by different metabolic mechanisms. This is very relevant from the functional point of view, considering the ability of sphingolipids to modulate the biological

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functions of PM-associated proteins, the membrane lateral organization (Prinetti et al. 2009).

However, the processes of neobiosynthesis and subsequent transport to the plasma membrane of the final compounds seem to require too much time to be used to modify the plasma membrane sphingolipid concentration and pattern in response to intra- and extracellular signals. In addition, it has been reported that the glycosphingolipid half life is short in neurons (Riboni et al. 1990, Prinetti *et al.* 2000). On the other hand, as discussed above, changes of sphingolipid pattern could be rapidly achieved upon activation of plasma membrane-associated enzymes as a consequence of ligand-triggered interactions, changes in their membrane lipid microenvironment or local proton concentration. Nevertheless, great care must be devoted also to the intracellular sphingolipid trafficking and to the recycling of partially degraded sphingolipids, which implies an important role of lysosomal activity.

Any derailment from the correct metabolic/traffic process can lead to an abnormal sphingolipid composition of the plasma membrane and, consequently, to an abnormal cell function modulation.

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SPHINGOLIPIDS AND LYSOSOMAL STORAGE DISORDERS (LSDs).

Lysosomal storage disorders are a wide group of genetic diseases due to reduced or absent enzymatic activity of catabolic lysosomal enzymes.

Altogether, more than 70 different LSDs are known, with an incidence of 1:200,000 - 1: 350,000 each. This makes the overall LSDs incidence around 1:5,000. Within LSDs, we count several sphingolipidoses due to genetic anomalies related to either the sphingolipid hydrolytic enzyme or the activator protein necessary for the enzyme activity. Glycohydrolases are exo-enzymes, and the incorrect catabolic process leads to the cellular primary accumulation of the enzyme substrate followed by a secondary accumulation of compounds likely due to lysosomal impairment (Fuller & Futerman 2018). In fact, secondary cell sphingolipid accumulation occurs probably in any cell with badly working lysosomes (see Table 1).

The main site of intracellular degradation of plasma membrane SL is represented by intra-endolysosomal vesicles (LVs) inside the late endosomal and lysosomal compartment, which are formed by an inward budding of the limiting endosomal membrane. Indirect evidence suggests that SL are sorted from the limiting lysosomal membrane to the intra-endolysosomal membranes: in fact, gangliosides artificially incorporated into the limiting lysosomal membrane are protected from degradation, while on the other hand they are

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promptly degraded at the lysosomal level upon normal plasma membrane turnover by endocytosis (Henning & Stoffel 1973). However, it is not known whether a certain amount of SL, after reaching the lysosome, remain associated with the limiting lysosomal membrane, thus escaping degradation. In rat liver, GM1 and GD1a can be formed by direct glycosylation of intravenously administered GM2 (Trinchera & Ghidoni 1990). In cultured neuroblastoma cells and neurons, exogenously administered GM1 can be converted into GD1a and GD1a into GT1b in the presence of chloroquine (Tettamanti 2004). These findings strongly suggest that a significant pool of ganglioside can escape lysosomal degradation and reach the Golgi apparatus, possibly by avoiding the sorting to degradation-prone LVs and remaining confined to the limiting lysosomal membrane. It is reasonable to expect that the magnitude of this pool might increase when for any reason sphingolipids are accumulated at the lysosomal level. An indirect support to this hypothesis comes from the finding that SM levels at the plasma membrane (as judged by lysenin staining) are 3.8 fold higher in cultured hippocampal ASM knock-out vs. wild type mice (Ledesma *et al.* 2011, Galvan *et al.* 2008).

The complexity of LSD, at the phenotypic and molecular level, particularly considering the aspects related to neuronal dysfunctions, clearly indicates that lysosomal impairment by itself might not necessarily be the central event in the pathogenesis of these diseases. Considering the importance of sphingolipid-dependent signal transduction and of sphingolipid-driven membrane organization (Santos & Preta 2018) in the physiology of neural cells, the obvious question is if and how primary or secondary accumulation of undegraded sphingolipids at the endosomal/lysosomal level could affect the composition/organization of the plasma membrane and of intracellular membranes limiting different organelles, with consequences on the pathology at the level of the nervous system.

Endosome/lysosomes can fuse with the plasma membrane. The exocytosis of multivesicular endosomes is likely the main mechanism leading to the release of exosomes, and has been extensively studied under this perspective (Harding *et al.* 2013), while the contribution of this event to a possible remodeling of the PM composition has received much less attention. On the other hand, it has been shown that lysosomes can fuse with the PM in response to the increase in intracellular Ca^{2+}, behaving as ubiquitous Ca^{2+}-regulated secretory vesicles (Rodriguez *et al.* 1997). Ca^{2+}-regulated exocytosis of lysosomes has been proposed as a mechanism for restoring the PM integrity after injury (Reddy *et al.* 2001) or for the insertion of pore-forming proteins such as certain bacterial

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toxins (Andrews *et al.* 2014). This process has been shown in several cellular models; however its relevance *in vivo* is still not confirmed, since only a few studies in tissues are available (Cheng *et al.* 2015, Jimenez & Perez 2017, Krause *et al.* 1994). The Ca^{2+}-sensing lysosomal protein synaptotagmin VII seems the crucial player for the regulation of the fusion of lysosomes with the PM upon influx of extracellular Ca^{2+} as a consequence of the loss of PM integrity (Reddy et al. 2001). In addition, PM repair by lysosomal fusion requires the rapid mobilization of lysosomes from the perinuclear region to the proximity of PM along microtubules via kinesin motors, or the transport of lysosomes, already present in the proximity of the PM, to the PM itself via actin filaments, events regulated by small GTPases, including Rab3a (Encarnocao *et al.* 2016) and Arl8b (Michelet *et al.* 2018). The exact mechanism by which lysosomal exocytosis promotes PM repair is still not clearly understood. The fusion could give origin to an intracellular "membrane patch" which could directly fuse with the wounded membrane leading to PM resealing. However, it is quite difficult to imagine a direct merge of the intracellular patch with the irregular wound. An alternative explanation could be the spontaneous resealing of the bilayer due to the reduction in membrane tension consequent to the exocytic process. A third, more complex possibility implies the enzymatic remodeling of the PM lipid composition as a consequence of the lysosomal fusion (reviewed in (Andrews et al. 2014)). Lysosomal acid sphingomyelinase (ASM) is secreted in the extracellular space or translocated to the cell surface upon membrane-damaging conditions. The hydrolytic activity of ASM on SM present in the outer leaflet of the PM can lead to the production of ceramide and of ceramide-enriched microdomains, triggering membrane dynamic remodeling and membrane invagination with consequent endocytosis, which in turn contributes to the removal of the lesion PM area. Lesion removal by ASM-regulated endocytosis would explain why Ca^{2+}-dependent lysosomal exocytosis, also repairs PM lesions caused by the insertion of pore-forming proteins. Besides ASM, other lysosomal hydrolytic enzymes acting on glycosphingolipids (β-hexosaminidases, sialidases, β-galactosidases, β-glucocerebrosidases) (Aureli et al. 2014a) can reach the PM after lysosomal exocytosis, potentially leading to the local production of ceramide or to a remodeling of the glycosphingolipid pattern of lipid raft/caveolar membrane domains. Indeed, glycolipid remodeling in caveolar domains could play a relevant role in this scenario, since caveolar endocytosis significantly contributes to PM lesion removal, as demonstrated by the observation that depletion of caveolin-1 by siRNA inhibited PM repair (Corrotte *et al.* 2013).

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All these proposed mechanisms imply the traffic of lysosomal components (lipids and enzymes associated with the limiting lysosomal membrane or soluble material present in the lysosomal lumen) to the cell surface. When considering the situation of LSD, this has profound implications that could explain the diversity of PM-generated alterations observed in these diseases (such as, merely as example, the ectopic dendritogenesis reported in GM2 gangliosidoses (Purpura & Suzuki 1976), as well as in α-mannosidosis (Siegel & Walkley 1994) and MPS type I (Walkley 2003), where secondary storage of GM2 is present). Most genes involved in lysosomal biogenesis and in lysosomal functions (including autophagy and lysosomal exocytosis) are under the control of the transcription factor EB (TFEB). The aberrant storage of uncatabolized material, common to all LSDs, induces the dephosphorylation of TFEB and its translocation to the nucleus, with the consequent activation of its target genes, leading to increased lysosomal biogenesis (a possible compensatory mechanism for the loss of lysosome functionality) (Sardiello *et al.* 2009). TFEB overexpression was sufficient to activate several key lysosomal genes and to induce lysosomal biogenesis. TFEB pathway was activated upon lysosomal storage of sucrose, a useful experimental model of lysosomal impairment, and TFEB activation was demonstrated in fibroblasts from mouse models of three different LSDs, Mucopolysaccharidoses types II (Muenzer *et al.* 2002) and IIIA (Hemsley & Hopwood 2005) (MPSII and MPSIIIA) and Multiple Sulfatase Deficiency (Settembre *et al.* 2007) (MSD). In addition, increased lysosomal biogenesis (even if not necessarily linked only to TFEB activation) was shown in several others LSDs or in their animal models (summarized in Table 2). Our group recently showed that, in sucrose-loaded human fibroblasts, reduced lysosomal activity led to the accumulation of undegraded sphingolipids both intracellularly and at the PM level, with consequent arrest of cell proliferation. Remarkably, this was accompanied by increased lysosomal biogenesis and lysosome fusion with the PM (Samarani *et al.* 2018). Obviously, increased lysosomal biogenesis in principle favors the traffic of lysosomal components to the cell surface with the above-described mechanisms implying lysosomal fusion with the membrane. This would explain the contribution of PM alterations to the pathogenesis of LSDs.

In addition to lysosome-mediated vesicular transport of lipids and lipid-specific enzymes, another form of lipid traffic has received much attention in recent times, and could contribute to alterations of lipid composition outside the lysosome in LSD, involving not only the PM but also intracellular membranes. It is becoming evident that intracellular organelles are physically and functionally interconnected by a complex network of contact

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sites between the membranes of different organelles (Membrane Contact Sites, MCSs) (reviewed in (Helle *et al.* 2013)). The endoplasmic reticulum (ER) establishes MCSs with almost all other cellular membranes, including the PM, lysosomes, early endosomes, endolysosomes and autophagic vacuoles, even if the best studied MCS is represented by the mitochondria-associated ER membranes (MAMs).

In additions, MCSs have been detected between lysosomes and mitochondria, lysosomes and the nuclear envelope, mitochondria and the Golgi membranes. The molecular organization of MCSs is complex and not fully understood, however it requires the tethering of both membrane protein complexes and lipids (likely organized as specialized lipid rafts) allowing to maintain the juxtaposed membranes in strict contact without undergoing fusion. Even if fusion does not occur, active exchange of lipids seems to take place, and one of the main supposed functions of MCSs seems the interorganelle lipid exchange. Since mitochondria are disconnected from vesicular traffic, exchange of lipids between ER and mitochondria at MAMs is likely crucial for the correct shaping of mitochondrial phospholipid pattern. Likely, lipid transport at MCSs involves lipid transport proteins able to extract lipids from a membrane and deliver it to the adjacent one. In the case of sphingolipids, at least two of such proteins, CERT (Kudo *et al.* 2008) and CLN8 (Winter & Ponting 2002), have been implicated as possible transporters of ceramide and other sphingolipids at MCSs. Topology of sphingolipids requires further care and according to their localization to one of the two membrane layers, together with the lipid transport proteins, flippase could be also necessary, this making the process of their transport at MCSs further complex. To date, still little is known about the contribution of altered lipid traffic at MCSs in LSDs. The best-studied example is GM1-gangliosidosis (reviewed in (Annunziata *et al.* 2018)). In the β-Gal$^{-/-}$ mice, the animal model of GM1-gangliosidosis, GM1 is massively accumulated in brain, and in particular in neurons, which undergo significant degeneration and death followed by micro- and astrogliosis. In neurons from β-Gal$^{-/-}$ mice brain, GM1 accumulation at the lysosomal level is accompanied by its abnormal redistribution from the lysosomes and the PM to the ER, where it accumulates prevalently at the level of MAMs. Increased GM1 levels in MAMs lipid rafts trigger a complex series of events leading to the formation and activation of a mitochondrial Ca^{2+} pore, and ultimately to the activation of the mitochondrial intrinsic apoptotic pathway. Interestingly, it has been shown that, on the other hand, changes in the structure of mitochondrial lipid rafts can play roles in the protection from neurotoxic stimuli (Garofalo *et al.* 2018).

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Taken together, all these pieces of evidence suggest that the hypothesis we proposed, that "the blockade of proper SL catabolism at the lysosomal level leads to the jamming of the overall flow of metabolites, with consequences on the SL composition in all cellular districts" (Piccinini *et al.* 2010) is still valid, however the "jamming" is likely much more complex than we would have expected, and involves highly specific events. Accumulation of SL in lysosomes in LSDs, either primary or secondary, eventually leads to a redistribution of SL in other subcellular districts. Considering the incredible complexity of the brain lipidome, the diversity of biological functions of different SL in different brain cell populations, and the variety of mechanisms potentially driving SL from lysosomes to other cellular membranes, with consequences of their organization, it is not surprising that neurological manifestations encompass a wide spectrum in LSDs. Addressing this diversity is one of the challenges of the basic research in this field.

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Table 1. Primary or secondary accumulation of (glyco)sphingolipids in LSDs ((Ballabio & Gieselmann 2009, Walkley & Vanier 2009, Hulkova *et al.* 2005, Piccinini et al. 2010) and references herein)

Disease	**Primary biochemical defect**	**Primary storage material**	**Secondary GSL storage**
Mucopolysaccharidoses (MPS)			
MPS I (Hurler and related)	α-iduronidase	dermatan sulphate, heparan sulphate	GM2, GM3
MPS II (Hunter)	iduronate-2-sulphatase	dermatan sulphate, heparan sulphate	GM2, GM3, LacCer
MPS IIIA (Sanfilippo)	heparan-*N*-sulphatase	heparan sulphate	GM2, GM3, GD2, LacCer
MPS IIIB (Sanfilippo)	*N*-acetyl-β-glucosaminidase	heparan sulphate	GM2, GM3, GD2
MPS IIIC (Sanfilippo)	acetylCoA: α-glucosamide N-acetyltransferase	heparan sulphate	GM2, GM3, GD2
MPS IIID (Sanfilippo)	*N*-acetylglucosamine-6-sulphatase	heparan sulphate	GM2, GM3, GD2
MPS VI (Maroteaux-Lamy)	arylsulphatase B	dermatan sulphate	GM2, GM3
MPS VII (Sly)	β-glucuronidase	heparan sulphate, dermatan sulphate, chondroitin sulphates	GM2, GM3
Sphingolipidoses			
Fabry	α-galactosidase A	Gb4Cer, Gb4-sphingosine	
Gaucher	β-glucosidase	GlcCer, GM1, GM2, GM3, GD3, Glc-sphingosine	
Globoid cell leukodystrophy (Krabbe)	galactocerebroside β-galactosidase	GalCer, Gal-sphingosine	LacCer, Gb3Cer, Gb4Cer,
Metachromatic leukodystrophy	arylsulphatase A	Sulfatide and other sulfolipids	GM2
Niemann-Pick A and B	sphingomyelinase	sphingomyelin	GM2, GM3, GlcCer, LacCer, Gb3Cer, Gb4Cer
GM1 gangliosidosis	β-galactosidase	GM1, GA1, lyso-GM1	GlcCer, LacCer, GM2, GM3, GD1a,
GM2 gangliosidosis (Tay-Sachs)	β-hexosaminidase A	GM2, GalNAcGD1a, GA2, lyso-GM2	
GM2 gangliosidosis (Sandhoff)	β-hexosaminidase A and B	GM2, GalNAcGD1a, globoside, GA2, lyso-GM2	
Salla	Membrane carrier mediating the egress of sialic acid from lysosomes		gangliosides
Others			
Niemann-Pick C	NPC1 and 2	cholesterol	LacCer
α-mannosidosis	α-mannosidase	mannose-containing oligosaccharides	GM2, GM3
Glycogenosis type II (Pompe)	α-glucosidase	glycogen	LacCer

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Table 2. LSDs with increased lysosomal biogenesis

LDSs	References
MPSI	(Ohmi *et al.* 2003)
MPSII	(Muenzer et al. 2002, Karageorgos *et al.* 1997)
MPSIIIA	(Hemsley & Hopwood 2005)
MPSIIIB	(Ohmi et al. 2003)
MPSVI	(Karageorgos et al. 1997)
MSD	(Settembre et al. 2007)
MLIV	(Venugopal *et al.* 2009)
Neuronal ceroid lipofuscinosis 3	(Pohl *et al.* 2007)
I-cell disease	(Kawashima *et al.* 2007)
Fabry	(Pereira *et al.* 2014)
Niemann-Pick A	(Gabande-Rodriguez *et al.* 2014)
Niemann-Pick C	(Kwiatkowska *et al.* 2014)
Sialidosis	(Wu *et al.* 2010)
Salla	(Karageorgos et al. 1997)
Fucosidosis	(Kondagari *et al.* 2015)
Pompe	(Karageorgos et al. 1997)

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LEGEND TO FIGURES

Figure 1. Comparison of the volume occupied by ceramide and by the hydrophilic head groups of selected sphingolipids.

Minimum energy structure of ceramide, of phosphocholine (head group of sphingomyelin), galactose (head group of galactosylceramide, cerebroside), II^3Neu5Ac-Gg_4 (head group of ganglioside GM1), IV^3Neu5Ac,II^3Neu5Ac_2-Gg_4 (head group of ganglioside GT1b) and IV^4GalNAc,IV^3Neu5Ac,II^3Neu5Ac-Gg_4 (head group of ganglioside GalNAc-GD1a). Oligosaccharide structures are aligned on the first sugar.

Figure 2. Metabolic pathways of sphingolipids. Representation of the main processes determining the membrane sphingolipid composition.

Ceramide is synthesized in the ER, transported to the Golgi where it is converted into SM and glycosphingolipids. Sphingolipids reach the plasma membrane by vesicular fusion and in very minor part transported by specific soluble proteins. Cell surface local structural changes can occur due to lactonization and to the enzymatic remodeling by cell surface associated hydrolases or synthases. Shedding of sphingolipids (Chigorno *et al.* 2005) as well as their uptake by the membranes were described (Saqr *et al.* 1993). The sphingolipid catabolism mainly occurs in lysosomes, from which sphingosine and some sugars can escape to be recycled (Ghidoni *et al.* 1987). Some primary and secondary lysosomes fuse with other membranes or exchange their membrane components (Trinchera *et al.* 1990).

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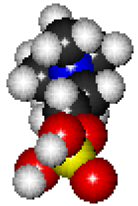

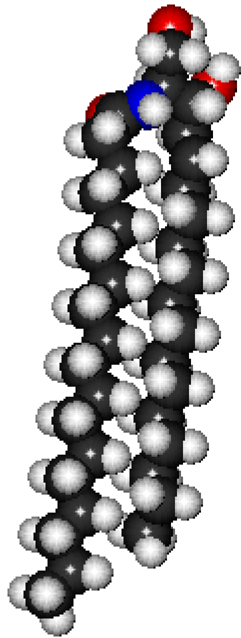

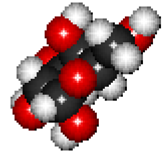

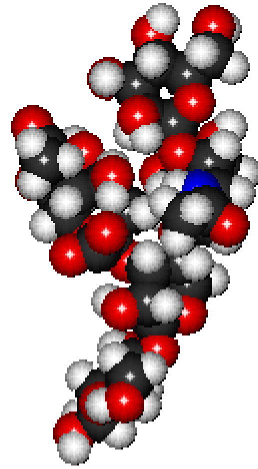

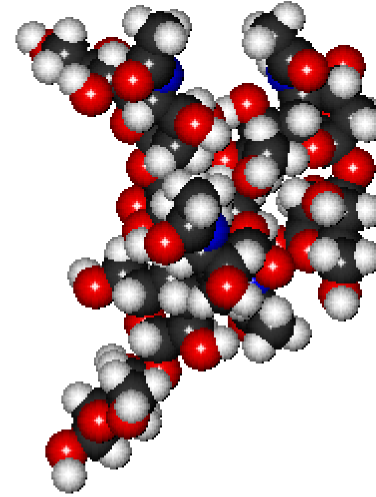

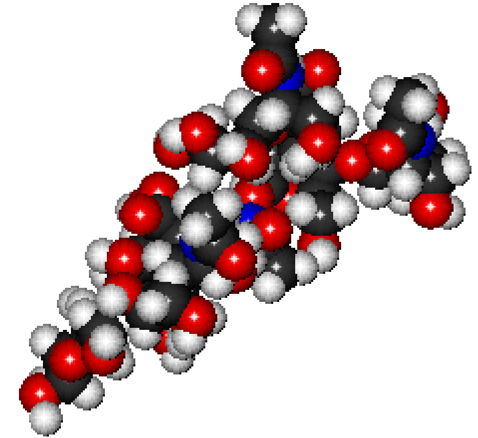

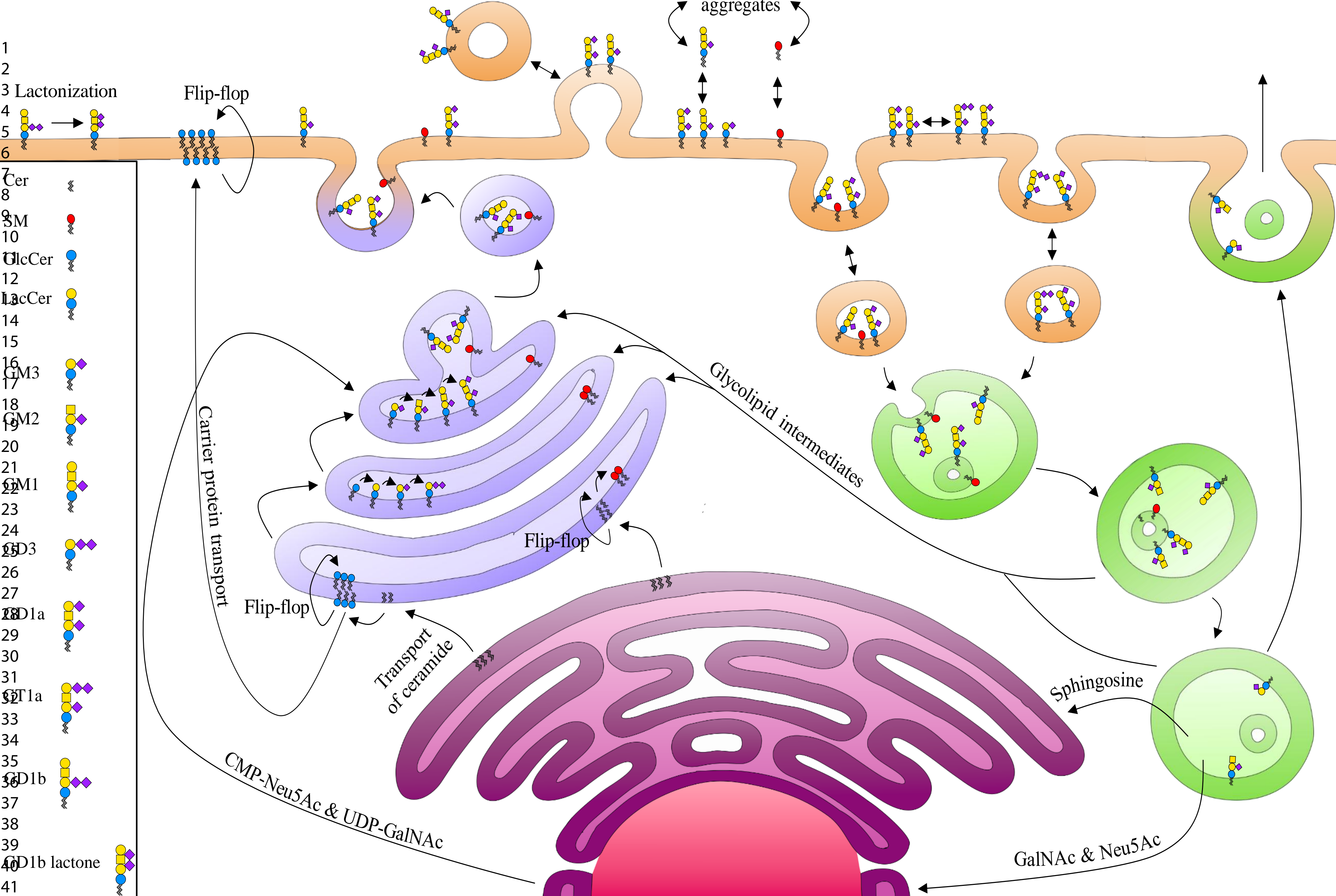
Sphingolipid aggregates
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Flip-flop
Carrier protein transport
Glycolipid intermediates
Flip-flop
Flip-flop
Transport of ceramide
Sphingosine
CMP-Neu5Ac & UDP-GalNAc
GalNAc & Neu5Ac
Cer
SM
GlcCer
LacCer
GM3
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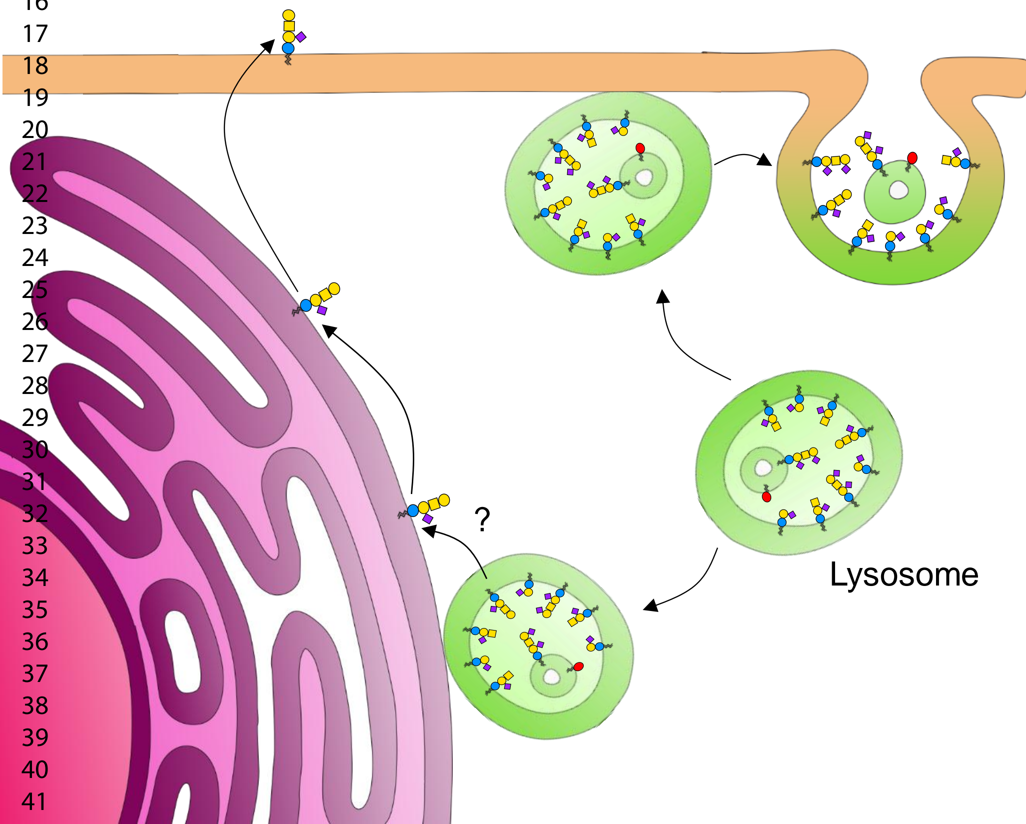

In this review article, we report on the sphingolipid chemical and biochemical properties and discuss some hypotheses about the possible links between sphingolipid incorrect catabolism and neurodegeneration.