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Review

Glycosphingolipid behaviour in complex membranes ☆

Alessandro Prinetti *, Nicoletta Loberto, Vanna Chigorno, Sandro Sonnino *

Center of Excellence on Neurodegenerative Diseases, Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, 20090 Segrate, Italy

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ABSTRACT

Glycosphingolipids, due to their tendency to form laterally separated liquid-ordered phases, possess a high potential for the creation of order in biological membranes. The formation of glycosphingolipid-rich domains within the membrane has profound consequences on the membrane organization at different levels, and on the conformational and biological properties of membrane-associated proteins and multimolecular protein complexes. In this review, we will discuss 1) how glycosphingolipids influence the lateral organization of biological membranes; 2) how glycosphingolipids influence the function of membrane-associated proteins.

Contents

1. Introduction

The notion that different levels of order exist in biological membranes is deeply rooted in cellular biology. The first level of ordered organization is the creation of the lipid bilayer, a consequence of the aggregational properties of complex amphipathic membrane lipids. The lipid bilayer provides a physical boundary between the cellular and extracellular environments, and a scaffold for molecules and molecular complexes that physically and functionally link these two environments [3]. The lipid bilayer is characterized by several basic properties that are relevant to its biological functions: the bilayer as a whole is a very stable structure, however it allows its components a certain degree of lateral motility. As a consequence of this fluidity, components of biological membranes can be arranged following a non-homogenous lateral distribution, leading to the creation of membrane areas ("domains") with a highly differentiated molecular composition and supermolecular architecture. The non-homogeneous lateral distribution of membrane components is made possible by the existence of lateral interactions stabilizing different membrane domains, and creating a second level of order in the organization of biological membranes. This level of order at the micron scale is particularly evident in polarized cells, such as polarized epithelial cells (where basolateral and apical membrane macrodomains can be distinguished) and neurons (characterized by the presence of somatodendritic, axonal and synaptic membrane macrodomains), but morphologically distinct domains specialized for particular

Abbreviations: EGFR, epidermal growth factor receptor; GSL, glycosphingolipid(s); IR, insulin receptor

☆ Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm [1], and the IUPAC-IUBMB recommendations [2].

* Corresponding authors. Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina, Via Fratelli Cervi 93, 20090 Segrate (Milano), Italy. Tel./fax: +390250330365.
E-mail addresses: alessandro.prinetti@unimi.it (A. Prinetti), sandro.sonnino@unimi.it (S. Sonnino).

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function are present at the membrane surface of virtually any cell type. Membrane macrodomains are in general characterized by the presence of specific subsets of proteins, and differential sorting and trafficking of proteins has been understood as one of the mechanisms responsible for the creation of polarized domains. In addition, lateral heterogeneity in membrane structure is present also at the sub-micron and nanometer scale, as indicated by the observation that even in membrane regions lacking a morphologically distinguishable architecture, certain proteins cannot undergo a free and continuous lateral diffusion, but rather are transiently confined to small domains ("microdomains") [4]. Obviously limitations to reciprocal diffusion are present for membrane proteins belonging to a multiprotein complex, such as those organized around membrane receptors or ion channels. More recently, to explain these limitations in lateral diffusion, theories such as the "membrane-skeleton fence" model have been proposed [5].

2. How do GSL influence the lateral organization of biological membranes?

Protein–protein interactions have been regarded for several years as the main factor responsible for the stabilization of membrane macro- and microdomains. However, in the last 10 years a role for membrane lipids in determining the lateral organization of the membrane has emerged, and the concept that certain membrane lipids, in particular glycosphingolipids (GSL), possess a high potential for the creation of order in biological membranes, is now well established [6]. Lipid membrane microdomains, or lipid rafts, areas in the membrane different in lipid composition from other membrane regions and characterized by a lateral organization dictated by the properties of their lipid components, have been involved in an incredible number of biological events [7–15]. However, the ability of glycosphingolipids to influence the organization and function of other components of biological membranes is still only partially understood, and it might only in part depend on their tendency to segregate, forming GSL-rich domains within the membrane. Lipid bilayers at physiological temperature (that is above the melting temperature for the most abundant glycerol- and sphingolipid molecular species) usually exist in a liquid-disordered (l*d*) phase characterized by high fluidity, in which the lipid acyl chains are disordered and highly mobile. Lowering the temperature below the melting point freezes the lipid acyl chains in an ordered gel phase with very limited freedom of movement. Membrane lipids can also exist in a third physical phase, the liquid-ordered (l*o*) phase. In the l*o* phase, the acyl chains of lipids are extended and ordered, as in the gel phase, but have higher lateral mobility in the bilayer The l*o* phase is stabilized by the presence of cholesterol, that fill the hydrophobic gaps between the phospholipid or glycolipid acyl chains [16,17]. The coexistence of lipids in different phases within the same model membrane was probably the first evidence leading to the concept of lipid domains. GSL-rich domains are likely more ordered than the l*d* phase, being in this regard similar to a l*o* or a metastable gel phase. The earliest evidence supporting the existence of lipid domains was obtained studying artificial membrane models (including glycerophospholipid vesicles or liposomes containing glycosphingolipids, sphingomyelin, ceramide or/and cholesterol, sphingolipid micelles, phospholipid bilayers on solid support, and lipid monolayers on an air/water interface or on solid support). Incomplete miscibility within a single phase, leading to lateral phase separation of complex lipids in phospholipid bilayers can be observed in binary mixtures of diacyl lecithins differing in chain length and/or saturation [18–20] and in ternary mixtures of palmitoyloleyl PC, dioleyl PC and cholesterol. However, sphingolipids, and GSL in particular, strongly differ from glycerolipids for their molecular structure and conformational properties (as discussed below), thus leading to a strong tendency to segregate within phospholipid bilayers. This tendency has been observed for non-glycosylated sphingolipids (including ceramide and sphingomyelin), for a wide range of neutral GSL molecular species, and for charged GSL, including sulfatides and gangliosides (Reviewed in [17]). Sphingomyelin undergoes lateral phase separation in dimyristoylphosphatidylcholine bilayers, and separation of ceramide-rich phases in phospholipid bilayers was observed for natural ceramides and for *N*-palmitoylceramide [20–24]. Comparing the behaviour of sphingomyelin and ceramide with the same fatty acid chain, a stronger tendency of ceramide for lateral phase separation, has been shown, suggesting that the interconversion of sphingomyelin and ceramide (due to the opposing actions of membrane-associated acid or neutral sphingomyelinases, or sphingomyelin synthase 2) could be responsible for rapid changes in the lateral organization of the membrane [21,22].

Gangliosides form high molecular weight micelles [25] in diluted aqueous solution. Using laser light scattering, the segregation of one ganglioside component with respect to the other has been observed in mixed micelles of two gangliosides with the same acyl chain but different hydrophilic headgroups. This observation emphasizes the importance of the geometrical properties of different GSL headgroups as determinant for their segregative tendency [26,27]. Lateral separation of gangliosides also occurs in one-component as well as in two-component, two-phase phosphatidylcholine bilayers (dielaidoylphosphatidylcholine: dipalmitoylphosphatidylcholine multilamellar liposomes, which exhibit laterally separated fluid- and gel-phase regions, more closely simulating the situation in biological membranes) and in phospholipid bilayers in the presence of cholesterol and/or sphingomyelin [28–37]. GM1-enriched domains are also present in sphingomyelin bilayers and the formation of separate GM1-enriched and cholesterol-enriched liquid-ordered phases was observed [38] in ternary sphingomyelin/GM1/cholesterol vesicles and in lipid monolayers (monomolecular lipid films formed at the air/water interface and transferred to silanized glass support) prepared from mixtures of synthetic lipids as well as by natural cell lipid extracts [39]. Thus, gangliosides display the ability to associate with a pre-existing ordered domain or to segregate in their own domains, that can be distinct from the cholesterol-enriched phase, as confirmed by the observation that the glycosynapse immunoseparated from B16 melanoma cells is enriched in GM3 ganglioside but not in cholesterol [93].

All these data from the study of artificial membrane models clearly demonstrate that sphingolipids form laterally separated phases characterized by reduced fluidity and hydrocarbon chain mobility. In other words they have the tendency to form "clusters" in fluid lipid bilayers, as these segregated structures were originally defined, or "lipid rafts", the term most widely used in the current literature. Segregation of membrane sphingolipids is responsible for the creation of less fluid membrane regions, where membrane-associated proteins can be confined, favouring lateral interactions between proteins that are segregated in the same lipid domain or preventing interactions between proteins that are associated with different domains. Moreover, proteins residing in sphingolipid- or GSL-rich areas have a higher probability to laterally interact with the confining lipids.

This behaviour implies the existence of lateral cooperative interactions between sphingolipid molecules. Again, studies conducted in model membranes allowed to elucidate the chemical and physicochemical properties of sphingolipids that drive their lateral organization as lipid membrane domains within biological membranes.

1) Due to the common hydrophobic ceramide backbone, all sphingolipids are characterized by the presence of a planar, relatively rigid system ranging from the amide group to the hydroxyl group on C3 disposed at the water/lipid interface of the bilayer [40]. The presence of an amide nitrogen, of a carbonyl oxygen and of a hydroxyl group, confers to sphingolipids to act both as donors and acceptors for the formation of hydrogen bonds [40]. This feature is unique for

sphingolipids among complex membrane lipids, and since the formation of each hydrogen bonds contributes 3–10 kcal to the lipid–lipid interaction, while van der Waals forces between hydrocarbon chains only contribute about 2–3 kcal per interaction, it is clear that the formation of a hydrogen bond network at the water/lipid interface strongly stabilizes the segregation of a rigid segregated phase enriched in sphingolipids.

2) In the case of GSL, another factor favouring their segregation within a biological membrane is represented by the presence of the bulky oligosaccharide hydrophilic headgroup. Theoretical calculations show that the volume occupied by the sugar GSL headgroup is much larger than that occupied by phosphocholine, the bulkiest headgroup present in phospholipids, requiring a bigger interfacial area to accommodate the amphipathic molecule in the bilayer. Phase separation with clustering of GSL in a phospholipid bilayer is a spontaneous process driven by the minimization of the interfacial free energy. The segregation of amphipathic molecules with a bulky hydrophilic headgroup implies the acquisition of a positive membrane curvature. The interfacial area increases with the size of the oligosaccharide chain, with a corresponding more positive membrane curvature and more pronounced segregation [25,41–52]. These predictions based on the geometrical parameters of the GSL molecules are experimentally confirmed by the observation that phase separation is present in mixed micelles of two gangliosides (GM2 and GT1b [26], or GD1b and GD1b-lactone [27]) with the same fatty acid composition and that the extent of ganglioside phase separation in glycerophospholipid bilayers depend on the surface area occupied by the GSL oligosaccharide chain, that is usually increasing with the number of sugar residues [31–33].

It has been suggested that GSL clustering could be facilitated and stabilised by the formation of carbohydrate–carbohydrate interactions, i.e. hydrogen bonds involving the GSL sugar head groups. Nevertheless, data proving evidence on direct side-by-side oligosaccharide interactions are not available. In fact, NMR spectroscopy on ganglioside micelles showed no NOE interactions between isolated GSL molecules, that should indicate inter-monomer carbohydrate–carbohydrate interactions [46]. In addition, no changes in the carbohydrate chain conformation were detected as an effect of the ganglioside-enriched environment. All this would exclude the presence of significant inter-molecular side-by-side interactions capable of altering the "conformational information" carried by the single saccharide chain of the ganglioside in the micellar model. This could be due to the large amount of water present in the hydrophylic layer [53]. Water is a natural component of the sugar shell being attracted by the hydrophylic character of sugars and by the necessity to avoid repulsion between the negative charged oligosaccharide. Calculations performed on GM2 micelles [26] indicated a difference of about 5 Å between the dry and hydrated micellar radius, suggesting that several molecules of water are interacting with the oligosaccharide chains. This is in agreement with data from calorimetric studies suggesting that each chain is surrounded by 40–70 water molecules [54]. A strong interaction between water and GM1 sugars, sialic acid and the inner galactose, was observed by NMR [46]. Water bridges between saccharides have been observed in hyaluronan where they were enough strong to determine and stabilize the tridimensional structure of the molecule [55]. Of course these results and considerations would exclude any direct inter-monomer side-by-side carbohydrate interactions at the level of cell membrane, but are in favor of a specific role of water in organizing a net of hydrogen bonds able to stabilized GSL clustering.

3) Membrane complex lipids, in particular glycerophospholipids, are highly heterogeneous in their fatty acid composition, bearing acyl chains with different number of carbon atoms and unsaturations. The presence of glycerophospholipids with a high degree of unsaturation ensures the membrane fluidity that is a necessary requirement for the functional properties of a biological membrane. The importance of unsaturated lipids in this regard is emphasized by their high levels in biological membranes from organisms adapted to life at extremely low environmental temperatures. However, a certain portion of complex lipids with saturated acyl chains is present in many biological membranes [56], and in some lipid classes, such as sphingomyelin and gangliosides (at least in the nervous system), saturated acyl chains (such as palmitic and stearic acid) are the main components. The presence of saturated acyl chains (that can be tightly packed with a high degree of order in the hydrophobic core of a bilayer) favours the phase separation of a rigid, liquid-ordered phase. In dielaidoylphosphatidylcholine: dipalmitoylphosphatidylcholine multilamellar liposomes, the lateral separation of fluid- and gel-phase regions has been observed [18], and in general phase separation occurs in binary mixtures of phosphatidylcholine molecular species differing in fatty acid chain length and/or saturation [31–37]. In the case of GSL, for GM1 it has been shown that distribution in the fluid phase of a phospholipid bilayer [35] is inversely correlated with the acyl chain length and directly correlated with the degree of unsaturation. Very long (≥C24) fatty acids are abundant in sphingolipids outside the nervous system. In the skin stratum corneum, where unusually long chain ceramides are the main lipid component, a high rigidity of the lipid phase was observed, that is mainly present as a crystalline, "solid" phase [57]. On the basis of neutron diffraction experiments on artificial membranes, it has been proposed that the organization of stratum corneum lipid bilayers could be highly stabilized by a partial interdigitation between the two leaflets [58]. The case of skin lipids is probably extreme, however a high concentration of C24 fatty acid chains has been reported in LacCer from human neutrophils, and a specific role of these long chain molecular species in the stabilization of GSL-rich domains due to lateral interactions between interdigitated hydrocarbon chains has been hypothesized [59,60]. Interdigitation of long chain fatty acid residues of complex membrane lipids might thus represent a further feature that favours the separation of liquid-ordered phases. On the other hand, interdigitation potentially not only influences the lateral organization of a single membrane leaflet, but somehow might be responsible for the tethering of the internal leaflet to the external one, possibly explaining how properties of GSL-rich domains present in the external leaflet might influence events localized on the cytosolic side of the membrane.

3. How do GSL influence the function of membrane-associated proteins?

As discussed in the previous section, segregation of membrane sphingolipids leads to the creation of laterally separated liquid-ordered phases that are less fluid than the surrounding membrane environment. The great interest for lipid-rich membrane domains, that gave rise to the complex and controversial discipline of raftology in the last two decades, derived from the observation that some membrane-associated proteins are highly concentrated in these domains, even if the overall protein content of these membrane areas is very low. It has been assumed that the trapping of certain proteins in lipid rafts might be somehow functional to their biological role [61]. Studies on model membranes, on detergent-resistant membrane fractions and in intact cells indicated without doubt that several classes of membrane-associated proteins display a strong preference for the association of lipid-rich membrane domains (reviewed in [62]). Among most common raft-targeting motifs are the presence of a GPI anchor (sometimes modified by acylation of the inositol head group or by replacement of the glycerolipid residue with ceramide) or of a lipid modification (tandem NH_2 terminal myristoylation/palmitoylation and double palmitoylation being especially efficient signals for targeting to lipid domains). Transmembrane proteins are in some cases also concentrated in lipid rafts, although modelling of transmembrane peptides indicated that in general targeting to liquid-ordered phases is disfavoured. Thus, the presence of a lipid modification (palmitoylation)

or of a specific targeting sequence within the hydrophobic or the extracellular/cytoplasmic domains is required. Finally, peripheral proteins can be associated or recruited to lipid rafts, possibly indirectly via interactions with raft-resident proteins.

Anyway, as mentioned above, it has been suggested, and in some cases proven, that the association with lipid-rich membrane domains can influence the function of a membrane protein. The question is, what are the mechanisms triggered by the association with lipid domains, or with lipids within lipid domains, that can affect the functional properties of a membrane protein. Several working hypotheses can be done in this regard. 1) The association of a protein with a liquid-ordered phase with reduced fluidity with respect the surrounding bilayer might represent a way to restrict the lateral motility of the protein, thus favouring more stable interactions with other proteins segregated in the same domains. In other words, the association with lipid rafts could represent a mechanism to facilitate the co-clustering of different membrane proteins. Against this simplistic view it can be argued that the surface density of proteins in lipid rafts is very low, and that in this case the limitations in lateral motility could indeed hamper protein–protein interactions. On the other hand, trapping of a protein within lipid rafts could prevent it from interacting with other proteins preferentially localized in fluid membrane regions: raft association might thus have an inhibitory significance for biological events requiring protein–protein interactions. 2) The association of a protein with a rigid membrane area could induce conformational changes in the polypeptide chain affecting its functional activity, independently from the formation of specific high affinity lateral interactions with other raft components. 3) Proteins concentrated in lipid rafts are favoured in their interactions with lipid components of the rafts. GSL, due to their complex oligosaccharide groups, are good candidates for specific lipid–proteins lateral interactions (it is worth to recall that *trans* interactions between the hydrophilic head groups of GSL and proteins located on different cellular membranes have been described [140]). Apart from the association with lipid rafts, the ability of GSL, and gangliosides in particular, to modulate the activity of membrane-associated proteins, such as receptor tyrosine kinases, has been widely documented (reviewed in [63,64]). Still to be elucidated in most cases remain the molecular aspects of GSL–protein interactions underlying the modulatory effect of GSL. The oligosaccharide chain of a GSL inserted in the plasma membrane could interact with a membrane protein via a) aminoacid residues belonging to the extracellular loops of the protein, if the conformation of the polypeptide chain allows them to be sufficiently close to the membrane surface; b) sugar residues in the glycans of a glycosylated protein, if the dynamics of the protein oligosaccharide chain allows the correct orientation toward the cell surface; c) the hydrophilic portion to the anchor in the case of GPI-anchored proteins (that is surely located in proximity of the extracellular surface of the membrane).

As mentioned above, early studies showed that many biological effects of gangliosides can at least in part be due to the modulation of several protein kinase systems [65–73]. The later observation that receptor and non-receptor protein kinases are highly enriched in lipid rafts suggested novel models for the interpretation of ganglioside-mediated signal transduction. In the following part of this review, we will describe some examples of protein functions influenced by the association with a GSL or a GSL-rich microenvironment in the plasma membrane.

3.1. GM3 and EGF receptor

Long time before the booming of raftology, the observation that exogenously added GSL can inhibit the growth of cultured cells led to the notion that the function of growth factor receptors can be modulated by gangliosides [65]. Epidermal growth factor receptor (EGFR) was identified as the target of the inhibitory action of GM3 [74]. GM3 inhibited EGFR autophosphorylation without competing with EGF for receptor binding [65,75,76], and without affecting receptor dimerization [77]. Other gangliosides exert a much smaller effect on EGFR, indicating the involvement of a highly specific interaction [74,78]. Studies on the purified human recombinant extracellular domain of EGFR indicated that the sialyllactose oligosaccharide is essential for ganglioside–receptor interaction, and that the substitution with any other sugar negatively affects the binding [78]. However, the molecular basis of this interaction has been only recently fully elucidated, emphasizing the importance of side-by-side carbohydrate–carbohydrate interactions between GM3 oligosaccharide and a N-linked glycan bearing multiple GlcNAc terminal residues on the receptor [79,80] (Fig. 1). GM3/EGFR interaction is facilitated by the enrichment of EGFR in classical ganglioside-enriched, cholesterol-sensitive, Triton X-100 insoluble membrane domains [81,82]. However, other GSL- and lipid raft-dependent factors can affect EGFR function. Caveolae and caveolin-1 are involved in the modulation of EGFR signalling [83,84] and EGFR is localized within a caveolin-rich fraction in A431 cells. However, EGFR-containing membrane fragments can be separated from caveolae [85,86]. In a keratinocyte-derived cell line, GM3 overexpression induced a shift of caveolin-1 to EGFR-rich membrane regions, allowing its functional interaction with the EGFR receptor, that caused inhibition of EGFR tyrosine phosphorylation and dimerization [87]. Thus, GM3 influences EGFR signaling by a second distinct molecular mechanism modulating EGFR/caveolin-1 association. Moreover, GM3 negatively regulates as well the ligand-independent cross-talk of EGFR with integrin receptor signaling, disrupting when accumulated in cultured cells, the interaction of integrin β1 subunit with EGFR [88].

3.2. GM3 and integrin receptors: the glycosynapse

The term “glycosynapse” has been introduced by S. Hakomori [89–91] to describe a membrane microdomain involved in carbohydrate-dependent adhesion (through GSL–GSL interactions or through GSL-dependent modulation of adhesion protein receptors, such as integrins). Type 3 glycosynapses are Brij 98-insoluble, Triton X-100-soluble [92] non-caveolar, cholesterol-independent GSL-rich membrane domains characterized by the presence of tetraspanins (tetraspan membrane protein superfamily members), such as CD9, CD81 and CD82. Tetraspanins are highly hydrophobic integral membrane proteins with four transmembrane stretches, strongly interacting with GSL [93]. On the other hand, tetraspanin association with integrin receptors [94] has been described. Both tetraspanin CD9 and integrin α3 or α5 receptor subunits are co-localized in glycosynapses (Fig. 2). In cell lines with high levels of GM3 ganglioside, CD9/integrin association is positively modulated in a GM3-dependent fashion and a multimolecular complex between GM3, tetraspanin and integrin α3β1 or α5β1 is stabilized, as shown by confocal microscopy and co-immunoprecipitation experiments [95–97]. The formation of integrin/CD9/GM3 complexes leads to the negative regulation of integrin-mediated signal transduction cell adhesion and tumor cell motility. In the presence of the GM3-dependent integrin/CD9 complex, the c-Src kinase Csk, responsible for the phosphorylation of c-Src at Tyr527, is translocated to the GSL-rich membrane domain with consequent c-Src inactivation, leading to reduced cell motility [97]. These findings indicate that integrin signaling machinery is modulated by GSL-containing membrane complexes localized within sphingolipid-enriched domains. Immunoseparated glycosynapses are highly enriched in GM3 and sphingomyelin, but not in cholesterol [98] (and glycosynapse-dependent signaling and tumor cell motility are not sensitive to cholesterol depletion [99]). The biological function of glycosynapses in regulating integrin-dependent tumor cell motility requires the presence of a specific GSL: exogenous addition of GM3, but not of GM1, is able to enhance the reciprocal association of α3 integrin and CD9 [97], and, in a reconstituted glycosynapse membrane model,

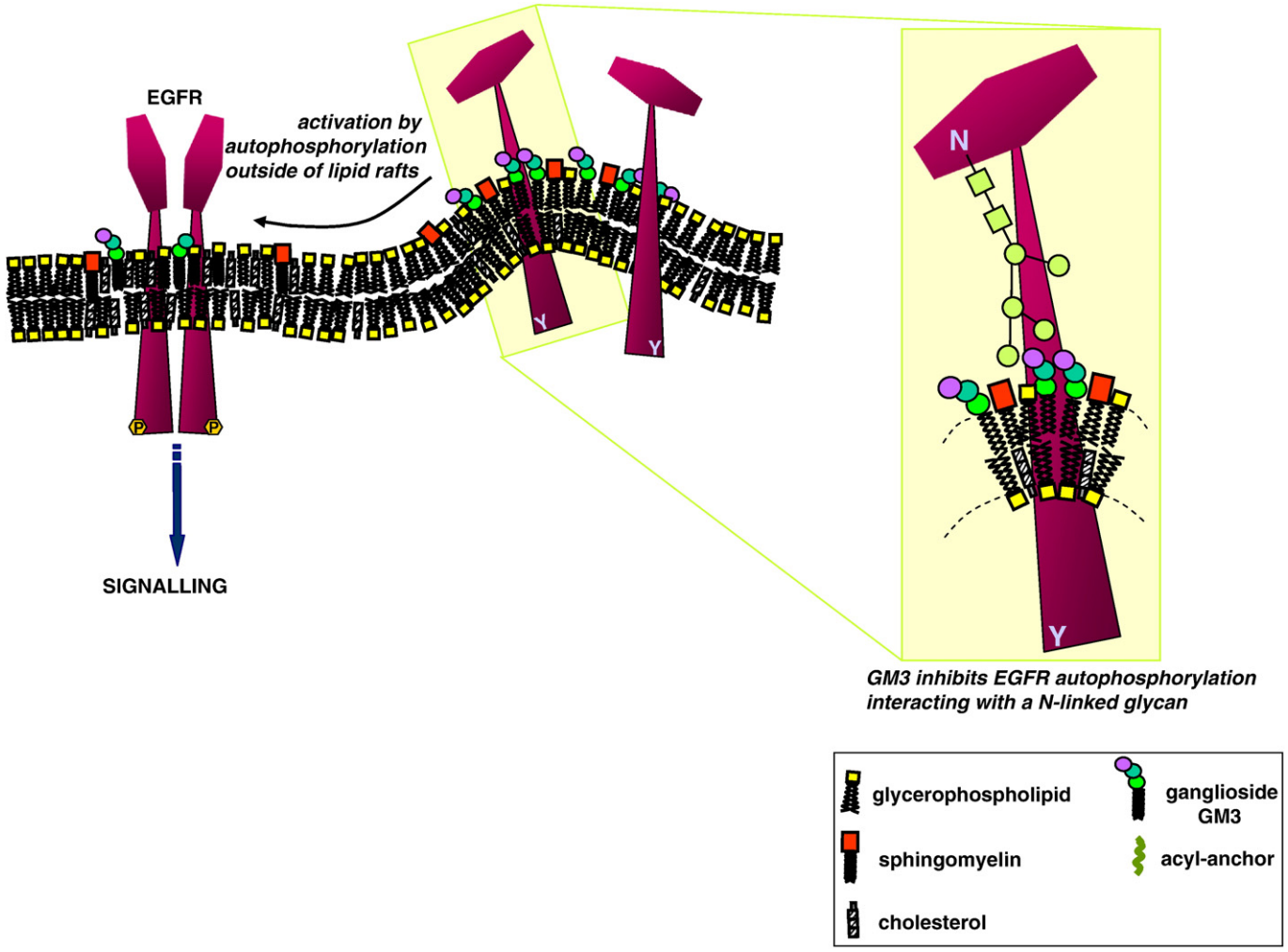

Fig. 1. GM3 and EGFR. EGFR is activated upon ligand binding outside of lipid rafts. In the presence of high GM3 levels, EGFR activation, autophosphorylation and dimerization is inhibited through a specific lateral interaction between the N-linked glycan of the receptor and the oligosaccharide chain of the ganglioside. Under these conditions, the shift of EGFR to GSL- and caveolin-1-rich membrane domains occurs, favouring further regulation of EGFR activity by complexes with caveolin-1.

c-Src phosphorylation was observed upon GSL-dependent adhesion, but it was not when GM3 was replaced with structurally related GSL, such as GM1, GD1a or LacCer [99], suggesting that a specific GSL–protein interaction involving the sialyllactose oligosaccharide chain is involved in glycosynapse-mediated signaling.

3.3. GM3 and insulin receptor

Insulin receptors (IR) are present in detergent-resistant membranes from normal adipocytes [100] and localized in caveolae in intact cells [101] (Fig. 3), where the β-subunit of IR interacts with caveolin-1 through a binding motif recognizing the scaffold domain of caveolin-1 [102]. Co-immunoprecipitation, cross-linking, florescence microscopy and FRAP experiments showed that IR can form distinct complexes with caveolin-1 and GM3 within lipid membrane domains [103]. The interaction between GM3 and IR is direct (IR can be cross-linked to a photoactivable GM3 derivative) and probably specific, since it is abolished by the addition of exogenous GM3 in co-immunoprecipitation experiments. The lateral interaction between GM3 and IR was abolished in IR mutants where the lysine residue at 944 was replaced with arginine, valine, serine, or glutamine, suggesting that an electrostatic interaction between the negatively charged sialyllactose chain of GM3 and the positively charged amino group of lysine 944, located in close proximity to the transmembrane domain sequence of IR, is essential for the formation of the GM3/IR complex (Fig. 3). In 3T3-L1 adipocytes, the induction of insulin resistance by treatment with TNFα was accompanied by the upregulation of GM3 synthase, leading to an increase of cellular GM3 [101,104], that accumulated in detergent-resistant membranes. In insulin resistance, the association of IR with GM3 was increased, while its association with caveolin-1 was decreased, indicating that the excess amount of GM3 in lipid membrane domains leads to the displacement of IR from the complex with caveolin-1 (Fig. 3), thus suggesting that the regulation of IR/caveolin-1 by GM3 could be responsible for the changes in insulin response in adipocytes [103].

3.4. Lactosylceramide and Lyn

Lactosylceramide (LacCer) is the most abundant GSL in human neutrophils [105] and it has a relevant role in neutrophil physiological functions [106,107]. LacCer on neutrophil cell surface binds several pathogenic microorganisms, and anti-LacCer monoclonal antibodies induce the phosphorylation of the Src family kinase with consequent chemotaxis, phagocytosis and superoxide generation [106–108]. LacCer in human neutrophils is co-localized in a detergent-resistant membrane fraction with Lyn [106] (Fig. 4), can be co-immunoprecipitated with Lyn and co-clusters at the cell surface with this protein [59,60]. The very long fatty acid C24:0 and C24:1 chains were the main components of LacCer (31.6% on the total fatty acid content) in the detergent-resistant membrane fraction (DRM) from neutrophil

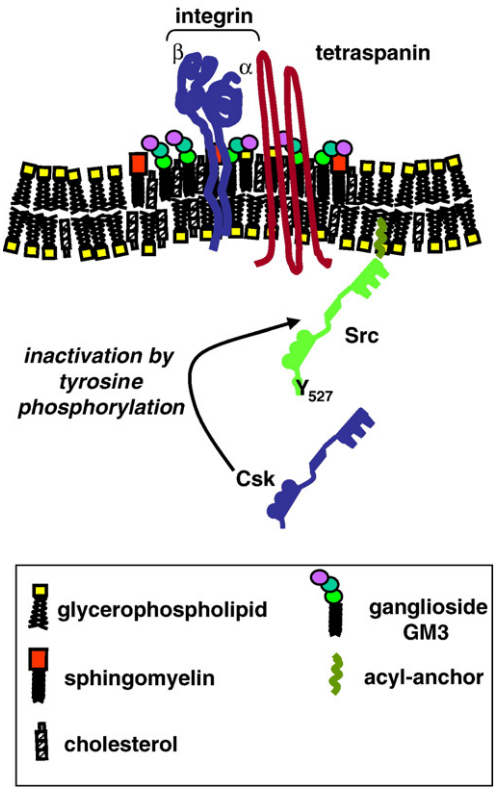

Fig. 2. GM3 and integrin receptors. In the glycosynapse, GM3 complexed with tetraspan membrane proteins (CD9 or CD82) and integrin receptor subunits participate in the control of cell motility. The GM3/CD9/integrin signaling complex formed in the presence of high cellular GM3 levels inhibits cell motility by recruiting Src inhibitor, Csk, thus keeping Src in a less active state.

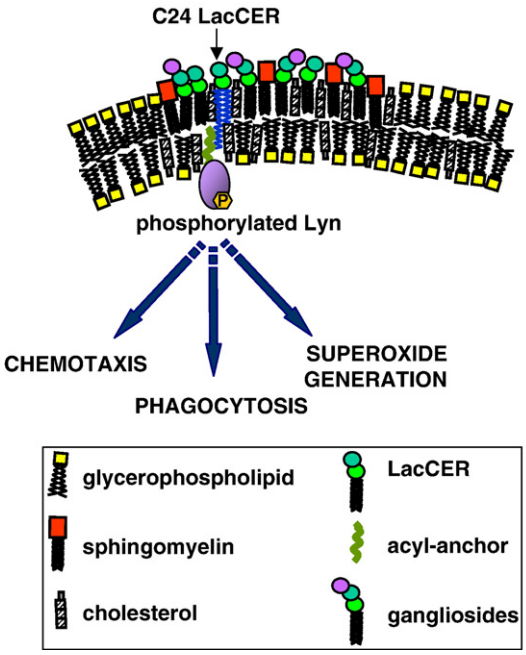

Fig. 4. LacCer and Lyn. In human neutrophils, Lyn is enriched in C24-LacCer-enriched domains and physically interacting with C24-LacCer, likely via van der Waals interactions between the LacCer hydrocarbon chains and those of Lyn. Lyn coupling with C24-LacCer is required for LacCer-mediated signal transduction, leading to superoxide generation, chemotaxis and phagocytosis.

plasma membranes [59,60]. Dimethyl sulfoxide (DMSO)-treated neutrophilic differentiated human promyelocytic leukemia HL-60 cells (D-HL-60 cells), an experimental model for the study of neutrophil function, are not able to respond with chemotaxis and superoxide generation to anti-LacCer antibodies, nor to phagocytose non-opsonized zymosans. As in neutrophils, LacCer is the main GSL in D-HL-60 cells, and it is enriched in detergent-resistant membrane, but it does not co-localize with Lyn. The main difference between D-HL-60 cells and neutrophils was found in the fatty acid composition of LacCer: in D-HL-60 cells, over 70% of LacCer in detergent-resistant membranes contains palmitic acid, and only 13.6% is represented by

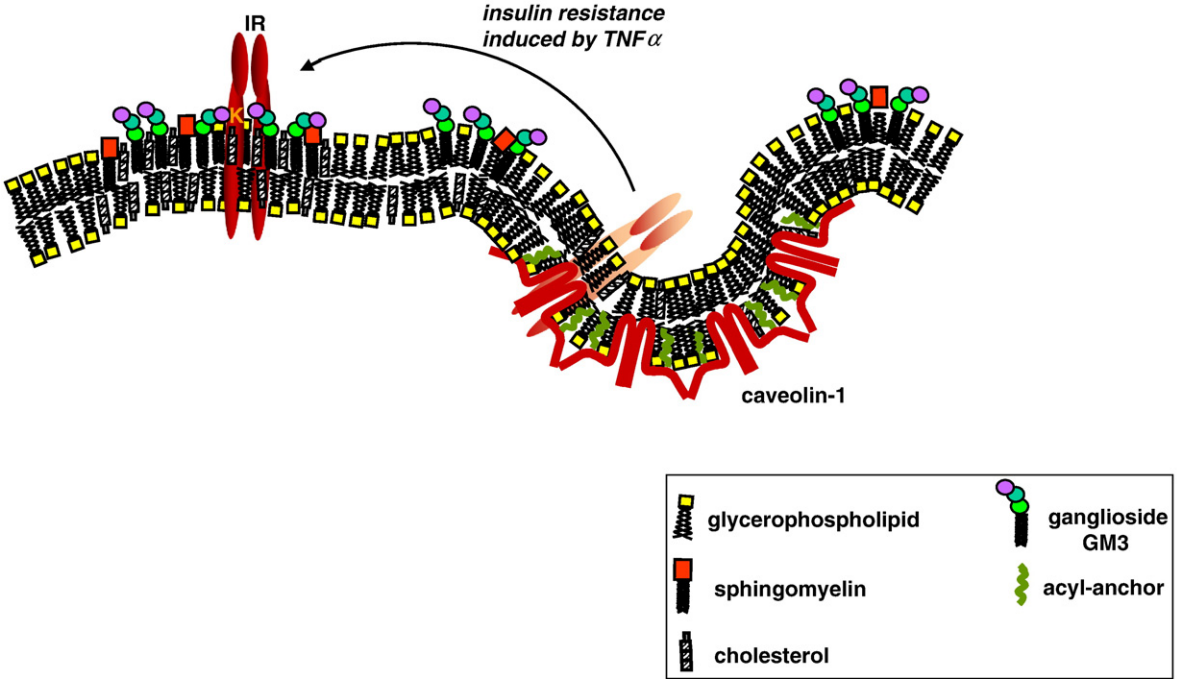

Fig. 3. GM3 and insulin receptor. In insulin-resistant adipocytes, GM3 synthesis is upregulated and the consequent accumulation of GM3 in detergent-resistant membranes induces the displacement of IR from the complex with caveolin-1 to the complex with GM3, inhibiting the downstream signal transduction. An electrostatic interaction between the sialyllactose chain of GM3 and lysine 944 in IR is required for the formation of the GM3/IR complex.

C24 species. When D-HL-60 cells were loaded with synthetic homogeneous LacCer molecular species, the added species became components of the detergent-resistant membrane fraction without affecting the overall LacCer cellular content. Loading with C24:0 or C24:1-LacCer, but not with shorter chain species, conferred to D-HL-60 cells the ability to migrate and to generate superoxides in response to anti-LacCer antibody [59,60], and induced CD11b/CD18-dependent phagocytosis of non-opsonized zymosans [108]. Moreover, in C24-LacCer loaded D-HL-60 cells, LacCer associated and clustered together with Lyn, suggesting that LacCer species with very long fatty acids are specifically necessary for the formation of membrane complexes and the functional coupling with Lyn. The physical and functional coupling of LacCer with Lyn could be due to direct van der Waals interactions between the GSL acyl chains (that, in the case of C24-containing species, are longer than the half hydrophobic thickness of the membrane bilayer) and those of Lyn. However, the sugar chain of LacCer also plays a relevant role in this interaction, since the addition of C24-GM1 did not affect the biological properties of D-HL-60 cells.

3.5. GM1 and Trk receptors

Pioneering works on ganglioside biology indicated that ganglio-series structures, the most represented in the nervous system, possess neuritogenic and neuronotrophic properties [109,110]. In particular for GM1, one of the main gangliosides in brain and neurons, many pieces of evidence, mainly based on the exogenous administration of this GSL, indicated that it exerts a positive effect on neuronal growth, differentiation and survival both in vivo and in cultured neurons, and a protective effect against neuronal injury, thus acting as a neurotrophic factor [111–114]. With its pleiotropic effect, GM1 in neurons seems to potentiate or to replace neurotrophins in their action [115]. This suggests that the GM1 neurotrophic effect could be mediated by Trk neurotrophin receptors. Indeed, early studies in PC12 cells showed that exogenous GM1 can stimulate Trk kinase activity, receptor autophosphorylation and dimerization [113,116], and that GM1 can specifically and tightly bind Trk [117]. The ability of GM1 (alone or in combination with neurotrophins) to activate TrkA, TrkB and TrkC receptors has been confirmed in other cell types [118–120], in brain

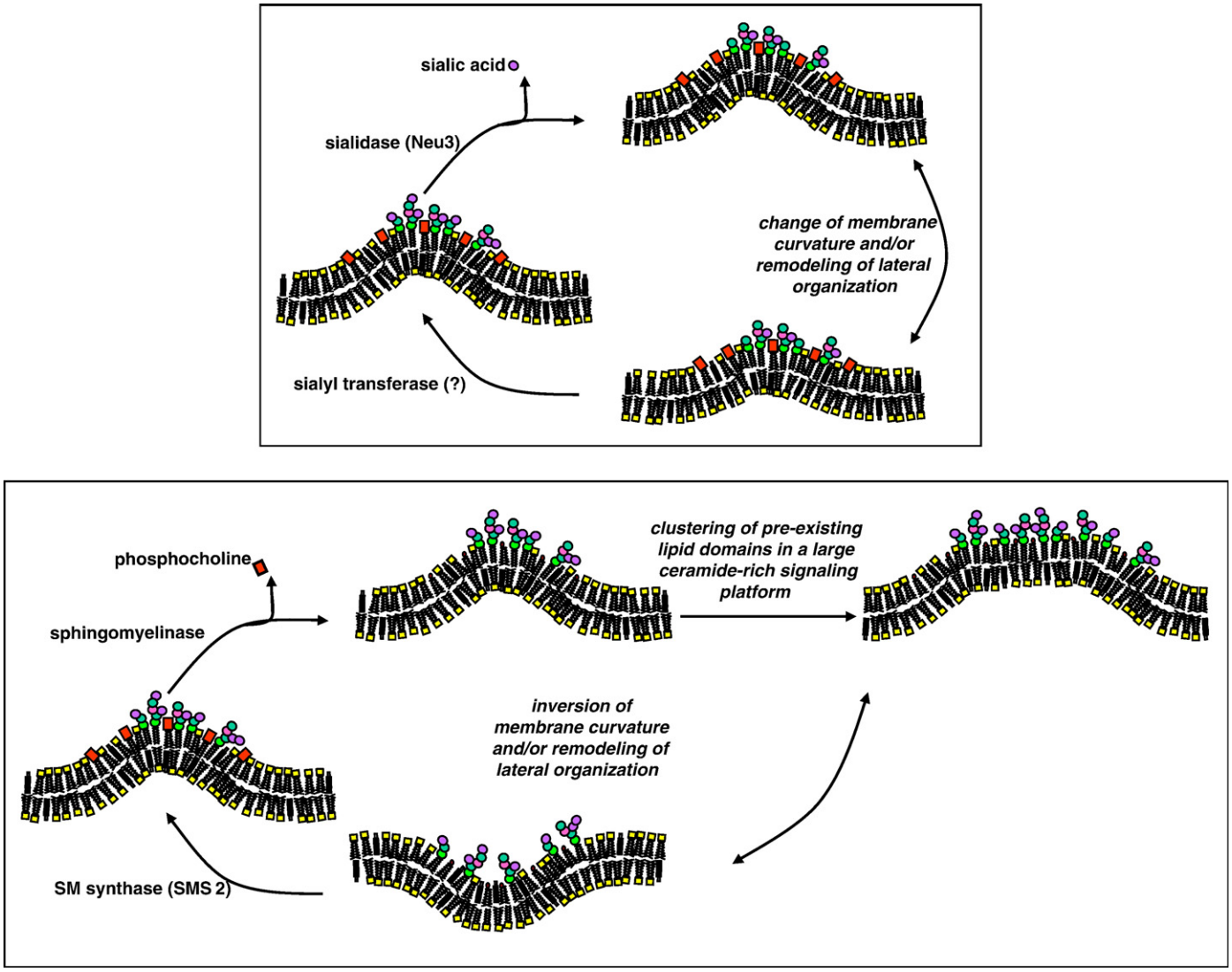

Fig. 5. Regulation of sphingolipid-enriched membrane domain composition by phosphorylation/dephosphorylation ("sphingomyelin cycle") and glycosylation/deglycosylation. The interconversion of ceramide and sphingomyelin due to the action of plasma-membrane resident sphingomyelinases and sphingomyelin synthase 2 leads to profound reorganization of the sphingolipid-enriched membrane domains. In particular, ceramide generation can lead to changes in the membrane curvature and vesiculation, or to the fusion of pre-existing small membrane domains into large ceramide-rich signaling platform [156]. GSL composition can be regulated by desialylation due to the plasma membrane-associated sialidase Neu3, and probably by membrane-resident glycosyltransferases, leading to changes in the geometry and organization of GSL-rich membrane domains and in specific GSL–protein interactions.

tissues [121] and in vivo [122,123]. A substantial amount of Trk receptors has been found to be associated with lipid rafts [124–129], and at least TrkB can be translocated to detergent-insoluble membranes from other cellular compartments upon stimulation with BDNF [124,125], suggesting that the receptor localization within GSL-rich membrane areas can be relevant for the regulation of their function. The ganglioside-specific, plasma membrane-associated Neu3 sialidase is abundantly expressed in the cerebral cortex and in the cerebellum [130], and at the subcellular level is enriched in lipid rafts [131], together with its putative substrates, polysialogangliosides. The activation of Neu3 might represent an efficient mechanism to locally increase the GM1 concentration, thus affecting Trk activity. In rat neurons, local activation of TrkA downstream to the action of Neu3 is essential in determining axon specification [132], and modulation in a positive or negative sense of Neu3 expression deeply affects neuritogenesis [133,134]. The extracellular portion of the receptor is essential for GM1-induced activation [120], and the glycosylation of Trk is required for the formation of Trk–GM1 complexes and for the targeting of Trk into GM1-enriched domains [135], suggesting that glycosylation could be a physiologically relevant mechanism for the regulation of Trk traffic and function, and that lateral interactions between the oligosaccharide chains of the receptor and of GM1 are responsible for receptor fine tuning. However, this hypothesis is challenged by some experimental observations, indicating that other mechanisms might be at least in part responsible for GM1 neurotrophic effect: 1) it remains to be elucidated whether GM1 treatment is able or not to induce the release of neurotrophins, triggering an autocrine loop in Trk receptor regulation. Rabin et al. showed that exogenous GM1 is able to induce the release of neurotrophin-3 (but not of other neurotrophins) from cultured fibroblasts and cerebellar granule cells [120]. On the other hand, no efflux of NGF, BDNF and NT3 has been observed upon incubation of slices from different brain regions with GM1 [121,122]. 2) GM1-induced neuritogenesis has been observed also in neurotumor cell lines (such as Neuro2a neuroblastoma cells) non responsive to NGF and lacking the expression of both high- and low-affinity NGF receptors [136,137]. In these cells, the neuritogenic effect of GM1 and other gangliosides seems to be linked to the activation of the non-receptor tyrosine kinase c-Src, highly enriched in GSL-rich membrane domains. 3) In neuroblastoma cells and in brain slices, signal transduction is triggered upon very short treatment with GM1. Under the experimental conditions used in these latter studies, GM1 remains loosely associated with the plasma membrane, with little or no serum- and trypsin-stable GM1 [116,132]. This would suggest that GM1 can interact with the exoplasmic portion of the receptor and trigger its activation without the need to be inserted in the same membrane, arguing against the involvement of lateral interactions between the GSL and the receptor [121,137]. 4) It has been shown that PC12 cells, the main cellular model used for the characterization of Trk-dependent GM1-induced signal transduction, indeed express little or no GM1, but high amounts of fucosylGM1. Thus, the possible role of fucosylGM1, and not GM1, as the endogenous modulator of Trk activity deserves further investigation [138].

4. The regulation of GSL composition of the plasma membrane

Clearly, according to the scenario described in the previous paragraphs, changes in the GSL composition of the plasma membrane in a certain cell type would lead to very important biological consequences, thus all mechanisms possibly contributing to these changes have a high functional significance. The classical view on sphingolipid metabolism implies the vesicular transport of neobiosynthesized sphingolipids from the endoplasmic reticulum and the Golgi apparatus to the plasma membranes (reviewed in [139]). Changes in the activities of enzymes of the biosynthetic pathway have been associated with the changes in GSL expression that are associated with biological events such as neoplastic transformation or neuronal differentiation. However, other mechanisms could be responsible for a local regulation of the GSL composition of plasma membranes or restricted plasma membrane areas (Fig. 5). 1) Both catabolic and biosynthetic enzymes for sphingolipids have been found associated with the plasma membranes. “Signalling” sphingomyelinases are resident in or translocated to the plasma membrane, being able to convert plasma membrane sphingomyelin into ceramide [140,141]. Conversely, a plasma membrane-associated sphingomyelin synthase enzyme activity (SMS2), genetically distinct from the Golgi enzyme, has been identified [142]. Thus, the sphingomyelin/ceramide ratio can be locally modulated possibly in response to physiological events, leading to profound consequence on the organization of sphingolipid-enriched membrane areas. In the case of GSL, a specific membrane-bound sialidase (Neu3) has been identified and cloned [130,143,144], and its role in modifying the ganglioside composition at the cell surface, acting as well on GSL molecules present on the surface of adjacent cells (i.e., in a “*trans*” fashion), has been proven [131,145,146]. Moreover, the presence of sialyltransferase activities at the cell surface has been also reported [147–151], Thus, glycosylation/deglycosylation cycles might be very important mechanisms responsible for rapid and possibly transient changes of the plasma membrane GSL composition, in analogy to that proposed for the “sphingomyelin cycle”. The presence of other active glycosylhydrolases, β-glucosidase, β-galactosidase and β-hexosaminidase [146,152], in the plasma membrane has been demonstrated, implying that local hydrolysis of GSL at the cell surface might represent a general mechanism for the control of GSL composition. 2) GSL can be released from the cell surface in different forms, including shedding vesicles [153–155], whose controlled release from specific glycolipid-enriched membrane areas, could represent a further way to modify the lipid membrane domain composition and organization.

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