# Chapter 10
# Gangliosides and Cell Surface Ganglioside Glycohydrolases in the Nervous System

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**Abstract** Gangliosides are a large group of complex lipids found predominantly on the outer layer of the plasma membranes of cells, and they are particularly concentrated in nerve endings. Their half-life in the nervous system is short, and their membrane composition and content are strictly connected to their metabolism. Their neobiosynthesis starts in the endoplasmic reticulum and is completed in the Golgi; catabolism occurs primarily in the lysosomes. However, the final content of gangliosides in the plasma membrane is affected by other cellular processes.

In this chapter structural changes in the oligosaccharide chains of gangliosides induced by the activity of glycohydrolases and in some cases by glycosyltransferases that are associated with plasma membranes are discussed. Some of the plasma membrane enzymes arise from fusion processes between intracellular fractions and the plasma membrane; however, other plasma membrane enzymes display a structure different from that of the intracellular enzymes. Several of these plasma membrane enzymes have been characterized and some of them seem to have a specific role in the nervous system.

**Keywords** Ganglioside • Glycosphingolipid • Glycohydrolases • Sphingolipid metabolism • Central nervous system • Neuronal differentiation • Neurodegeneration

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## 10.1 Gangliosides

Gangliosides are glycosphingolipids (GSLs) that contain one or more sialic acid residues. They are components of the external layer of all animal cell plasma membranes and are particularly abundant in brain where they are tenfold higher than in extra-nervous tissues, representing one-twelfth of the outer layer of glycerophospholipids. Neuronal gangliosides are lipids with strong amphiphilic character due to their acidic and, in general, large saccharide head group and double-tailed hydrophobic moiety. The lipid moiety of gangliosides, as well as that of all sphingolipids, is ceramide (Fig. 10.1a), which consists of a long-chain amino alcohol connected to a fatty acid by an amide linkage. As a peculiar characteristic of the nervous system, the amino alcohol can be either 2*S*,3*R*,*4E*,2-amino-1,3-dihydroxy-octadecene, known as sphingosine, or 2*S*,3*R*,*4E*,2-amino-1,3-dihydroxy-eicosene, known as eicosasphingosine. The ratio between the two long-chain alcohols is variable, with eicosasphingosine barely detectable in the brains of fetuses but progressively increasing with age to become the major species in the elderly. A few percent of the saturated species, sphinganine and eicosasphinganine, have also been identified (Valsecchi et al. 1996; Valsecchi et al. 1993).

In nonnervous tissue gangliosides exhibit heterogeneity in their acyl chains, with a very long fatty acid moiety often being a major component of the ceramide structure. In gangliosides of the nervous system, stearic acid is the most common fatty acid accounting for 90–95 % of the total fatty acid content. This characteristic of neuronal gangliosides may be necessary for neuronal membrane plasticity.

The oligosaccharide chain of neuronal gangliosides varies widely due to the neutral sugar content and number of sialic acid residues. This, together with the heterogeneity of the ceramide moiety, makes gangliosides a very large family of compounds. Table 10.1 shows the main ganglioside structures found in the human nervous system, together with trivial and commonly used abbreviations.

Sialic acid is the name that identifies all the derivatives of 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid, or neuraminic acid. Among the sialic acids, the 5-*N*-acetyl (Fig. 10.1b), the 5-*N*-acetyl-9-*O*-acetyl (Fig. 10.1c), and the 5-*N*-glycolyl derivatives (Fig. 10.1d) are the most common. Healthy humans have only the first two sialic acids, in the ratio of 9:1 (Kamerling and Vliegenthart 1975). Also ganglioside lactones, containing polysialyl chains where the sialic acids are linked together with ketosidic and ester linkages (Fig. 10.1e), have been found in human brains (Riboni et al. 1986).

## 10.2 Gangliosides and Membrane Organization

Gangliosides are enriched in confined areas of the plasma membrane known as "lipid rafts" (Sonnino et al. 2006). Lipid rafts are enriched in sphingolipids and cholesterol with respect to glycerophospholipids and contain about 1–4 % of the

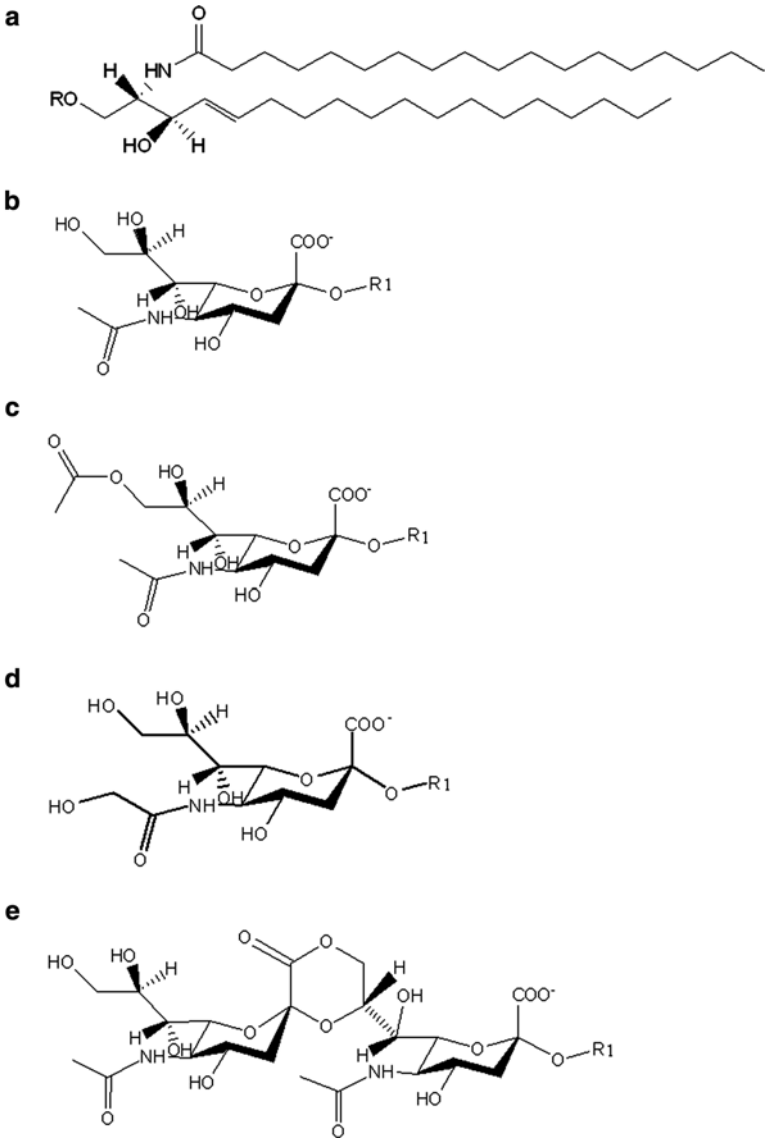


**Fig. 10.1** Chemical structures of ceramide and the sialic acids. (**a**) Ceramide, a long-chain amino alcohol (sphingosine base) connected to a fatty acid by an amide linkage. R: phosphocholine, glucose, or oligosaccharides. (**b**) Sialic acid is the name that identifies all the derivatives of 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid, or neuraminic acid. (**c**) 5-acetamido-9-*O*-acetyl-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid. (**d**) 3,5 dideoxy-5-glycolamido-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid. (**e**) Sialic acids linked together with ketosidic and ester linkage. R1: saccharides or oligosaccharide chain

**Table 10.1** The main oligosaccharide series in the human nervous system

| Structure of the oligosaccharide chain | Series |
|---|---|
| β-Gal- | Gal |
| β-Gal-(1-4)-β-Glc- | Lac |
| β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc- | $Gg_3$ |
| β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc- | $Gg_4$ |
| β-GalNAc-(1-4)-β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc- | $Gg_5$ |
| β-Gal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc- | $nLc_4$ |
| The main gangliosides from the human nervous system | |
| Svennerholm nomenclature | IUPAC-IUB nomenclature |
| GM4 | Neu5AcGalCer |
| GM3 | $II^3$Neu5AcLacCer |
| GD3 | $II^3(Neu5Ac)_2$LacCer |
| GM2 | $II^3Neu5AcGg_3Cer$ |
| GD2 | $II^3(Neu5Ac)_2Gg_3Cer$ |
| GM1 | $II^3Neu5AcGg_4Cer$ |
| 3′-LM1 | $IV^3nLc_4Cer$ |
| GD1a | $IV^3Neu5AcII^3Neu5AcGg_4Cer$ |
| GalNAc-GD1a | $IV^3Neu5AcII^3Neu5AcGg_5Cer$ |
| GD1b | $II^3(Neu5Ac)_2Gg_4Cer$ |
| GD1b-lactone | $II^3$[Neu5Ac-(2-8,1-9)-Neu5Ac]$Gg_4Cer$ |
| GT1b | $IV^3Neu5AcII^3(Neu5Ac)_2Gg_4Cer$ |
| *O*-Acetyl-GT1b | $IV^3Neu5AcII^3$[Neu5,9$Ac_2$-(2-8)-Neu5Ac]$Gg_4Cer$ |
| GQ1b | $IV^3(Neu5Ac)_2II^3(Neu5Ac)_2Gg_4Cer$ |
| *O*-Acetyl-GQ1b | $IV^3(Neu5Ac)_2II^3(Neu5Ac)_2Gg_4Cer$ |

total protein content of the cell (Sonnino et al. 2006). Many lipid raft proteins are involved in cell signaling, and this led to the concept that ganglioside–protein interactions are instrumental in signal transduction and cell function. Protein properties might be affected by specific interactions but could also be modified by the physicochemical properties of the membrane which are determined by the lipid pattern, lipid amphiphilic and geometric properties, and lipid organization. Gangliosides, with their complex and bulky oligosaccharide structures, need a larger interfacial area than that required by glycerolipids. Phase separation with clustering of GSLs in a phospholipid bilayer is a spontaneous process driven by minimization of the interfacial free energy. The segregation of amphiphilic molecules with a bulky hydrophilic head group implies acquisition of a positive membrane curvature. The interfacial area increases with size of the oligosaccharide chain, along with a more positive membrane curvature and more pronounced segregation. The geometric properties of individual gangliosides inserted into the membrane depend primarily on the structural features of their oligosaccharides (Sonnino et al. 1994) and to a lesser extent on those of ceramide. Any variation in either the head group and/or the ceramide portion can have marked effects on the membrane surface, particularly on lipid rafts, where gangliosides are highly enriched. The dramatic effect on

membrane curvature/organization seen when sialidase was used to convert the disialoganglioside GD1a to monosialoganglioside GM1, in an artificial membrane (Del Favero et al. 2011), supports the hypothesis that the effect of sequential hydrolysis of gangliosides to ceramide by coordination of plasma membrane-associated GSL hydrolases, as it occurs in senescent neurons and during neurodegeneration, should be much greater (Aureli et al. 2011c). Due to its two hydrocarbon chains, ceramide is very hydrophobic and almost insoluble in an aqueous solution. In a membrane it can be considered amphiphilic due to the primary hydroxyl group and the amide planar linkage located at the water–lipid interface. It is claimed that when a large amount of ceramide is produced from complex sphingolipids, it rapidly segregates forming macrodomains instrumental for cell signaling (Gulbins and Grassme 2002). Ceramide has a very high packing parameter suitable for negative curvature. The removal of the head group from sphingomyelin leads to ceramide-enriched endocytic vesicles, in artificial membranes (Holopainen et al. 2000). In a natural membrane this process would require rearrangement of the membrane, with exclusion of some components and sorting of others. In this context, the original lipid–protein interactions, or the forces exerted by the lipid environment on the protein conformation, would change with concomitant modifications of protein biological properties.

## 10.3 Metabolic Pathways of Gangliosides

The dynamic plasma membrane ganglioside content and pattern are determined by neobiosynthesis, catabolism, and complex trafficking into and out of the cell. Change in any of these pathways can lead to alterations in plasma membrane ganglioside content that can affect neuronal differentiation and neurodegeneration. A general scheme for GSL metabolism is shown in Fig. 10.2.

De novo biosynthesis of GSLs requires ceramide which is synthesized in the endoplasmic reticulum. In contrast to other cells, neurons have two different serine acyl-CoA acyltransferases: one specific for palmitoyl-CoA and the other for stearoyl-CoA. They are expressed in different ratios and in a spatiotemporal manner during neuronal development (Chigorno et al. 1997a) and are necessary for the first step in the synthesis of sphinganine and eicosasphinganine, respectively. The biosynthetic process leading to ceramide is shown in Fig. 10.3. Ceramide can be transported to the Golgi apparatus, the site of ganglioside synthesis, by either vesicular or non-vesicular (protein mediated) trafficking (Olayioye and Hausser 2012); neobiosynthesized gangliosides are transferred to the plasma membrane by a vesicle transport system, becoming external leaflet components. Catabolism of sphingolipids occurs in lysosomes, from which less complex products obtained in the degradation pathway can escape (Kolter and Sandhoff 2005). In neurons, only a minor portion of endocytic vesicles become lysosomes, while the rest rapidly reassociate with the plasma membrane. Among the more hydrophilic gangliosides, only a minor portion is released from the plasma membranes into the extracellular environment

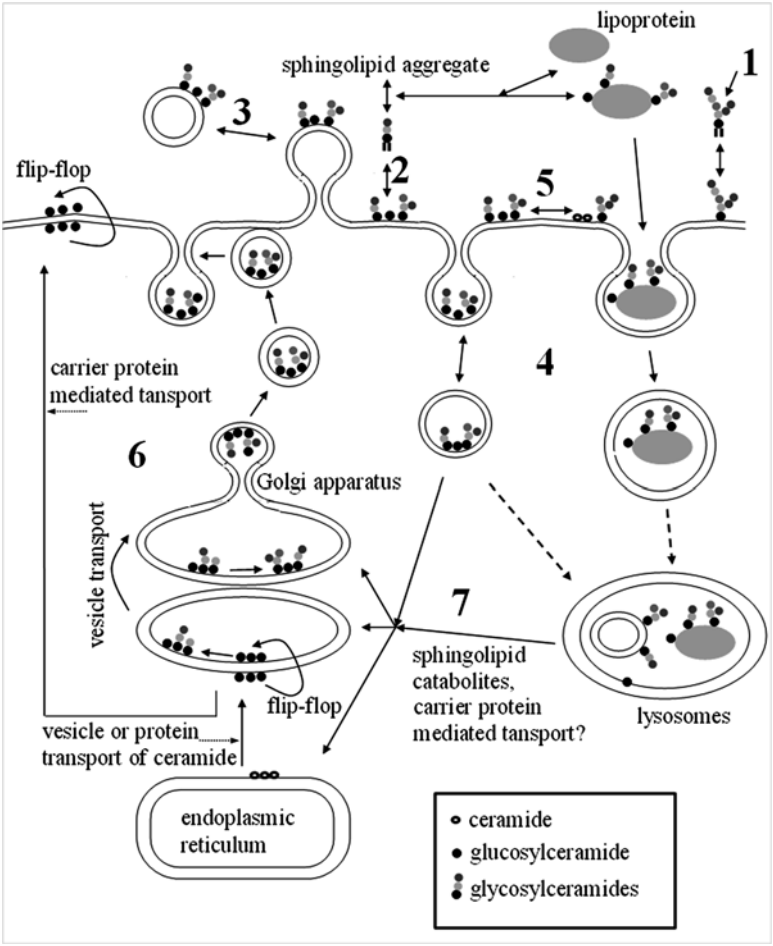


**Fig. 10.2** Scheme depicting glycosphingolipid metabolism. Different metabolic pathways involved in changing plasma membrane glycosphingolipids composition. 1—plasma membrane uptake of extracellular glycolipids shed by different cells; 2—shedding of glycolipid monomers, some directly reenter the membrane, while others interact with the extracellular proteins or lipoproteins and are subsequently taken up by the cells and catabolized into lysosomes; 3—release of glycolipid-containing vesicles from the plasma membrane; 4—membrane endocytosis followed by sorting to lysosomes and lysosomal catabolism; 5—biosynthetic modifications by plasma membrane-associated glycosyltransferases and glycosidases; 6—neobiosynthesis of glycosphingolipids and their transport to the cell surface; 7—recycle in the biosynthetic pathway of partially catabolized molecules

(Chigorno et al. 2006). Sphingolipids present in the extracellular milieu are, at least in part, taken up by cells, becoming components of membranes where they may modify their composition, or they may be directly sorted to the lysosomes (Saqr et al. 1993). Finally, recent evidence indicates that both sphingolipid catabolic

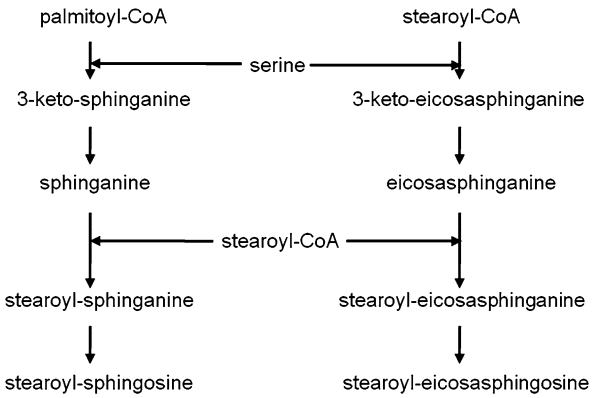


**Fig. 10.3** Biosynthetic pathway for brain ceramide. Ceramide is synthesized in the endoplasmic reticulum. Neurons have two different serine acyl-CoA acyltransferases: one specific for palmitoyl-CoA and the other for stearoyl-CoA. They are expressed in different ratios and in a spatiotemporal manner during neuronal development and are necessary for the first step in the synthesis of sphinganine and eicosasphinganine, respectively

(hydrolases) and biosynthetic (transferases) enzymes are associated with the plasma membrane where they may act on membrane components (Aureli et al. 2011b).

While steps in the biosynthesis of GSLs are now well defined, little is known about how it is regulated. It is generally assumed that it is primarily regulated at the transcriptional level by control of expression of glycosyltransferases and/or transporter proteins. Indeed, changes in cellular GSL pattern such as that seen during neuronal development and upon oncogenic transformation and acquisition of drug resistance by tumor cells are paralleled by changes in expression of the corresponding glycosyltransferases. However, it is also possible that differences in intracellular metabolic flow and/or half-life of each GSL influence the GSL pattern (Veldman et al. 2002). In fact, regulation of intracellular trafficking may be as important as control of enzyme expression in determining the final GSL composition of the plasma membrane. Reliance of cells on neobiosynthesis and subsequent transport of the GSLs to the plasma membrane could require too much time to modify the plasma membrane GSL composition in response to extracellular signals. In addition, even though the half-life of GSLs may be short in neurons (Prinetti et al. 2001), it is long or very long in the majority of cells. In fibroblasts, where the half-life of gangliosides is long (Chigorno et al. 1997b), their major loss is by shedding into the extracellular environment (Chigorno et al. 2005), not catabolism. It has been determined that up to 7–8 % of the total cell sphingolipids are shed every day by cells in culture (Chigorno et al. 2006), making neobiosynthesis of GSLs a necessity.

The fact that some enzymes involved in GSL metabolism are found in the plasma membrane modifies the generalized concept that glycohydrolases are only in the

lysosomes and glycosyltransferases are only in the Golgi. The association of enzymes needed for GSL metabolism with the plasma membrane, accumulating information about their activity on natural substrates in living cells, along with identification of the presence of pairs of enzymes, like sialidase and sialyltransferase, able to catalyze opposing reactions, suggest that changes in plasma membrane GSLs could occur rapidly in response to different conditions. These changes would be dependent only on kinetic properties of the enzymes, which can change very rapidly in response to ligand-triggered interactions.

## 10.4 Plasma Membrane-Associated Enzymes and Ganglioside Pattern

Enzymes involved in GSL metabolism that are associated with the plasma membrane include sialidase and sialyltransferase, β-hexosaminidase, β-galactosidase, and β-glucosidase (Fig. 10.4). In gangliosides sialic acid is usually linked by an α-linkage to the C3 position of galactose or to the C8 position of another sialic acid residue. Four different sialidases capable of catalyzing release of ganglioside sialic acid moieties have been identified. Neu1 is the lysosomal enzyme, known to be needed for catabolism of sialo-compounds; Neu2 is cytosolic and Neu4 is mitochondrial, but their role in vivo is not clear. Neu3 is associated with the plasma membrane and seems to be involved in functional processes. Small amounts of Neu1 and Neu2 have been also found in association with the plasma membrane.

Publications in the 1970s and 1980s described the association of sialidase (Schengrund and Rosenberg 1970; Tettamanti et al. 1972; Tettamanti et al. 1973; Tettamanti et al. 1975) and sialyltransferase (Preti et al. 1980) with synaptosomal membranes. These observations led to the hypothesis that a sialylation–desialylation cycle existed for gangliosides at the plasma membrane level and might be involved in the function of gangliosides in neurons. The sialylation–desialylation cycle

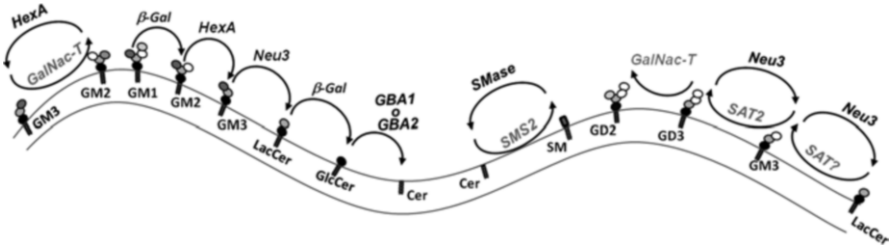


**Fig. 10.4** Schematic representation of the glycosyltransferases and glycohydrolases associated with the cell surface. The presence of different glycosyltransferases and glycohydrolases at the cell plasma membrane allows the in situ modification of the cell surface glycolipid composition. The availability of a series of couples of enzymes catalyzing the same reaction in opposite directions extends the concept of cycle, originally reported for the sphingomyelin, to several other sphingolipids. HexA, hexosaminidase A; GalNac-T, UDP-N-UDP-N-acetylgalactosaminyltransferase; β-Gal, β-galactosidase; SMase, sphingomyelinase; SMS2

predates by 20 years the elucidation of the "sphingomyelin cycle" (Venable et al. 1995), in which the two enzymes sphingomyelinase and sphingomyelin synthase modulate cell proliferation and apoptosis through the level of ceramide. The existence of a plasma membrane-associated sialidase distinct from the lysosomal enzyme was first suggested by enzymatic and immunological studies (Schengrund et al. 1976; Miyagi et al. 1990a; Miyagi et al. 1990b; Schneider-Jakob and Cantz 1991; Kopitz et al. 1994), as well by metabolic studies (Riboni et al. 1991; Kopitz et al. 1997a); a membrane-bound sialidase was purified from human brain grey matter (Kopitz et al. 1997b) and from bovine brain (Hata et al. 1998; Oehler et al. 2002). In 1999, the existence of a specific membrane-associated sialidase, coded as Neu3, that was distinct from other known sialidases, was unambiguously proven by the cloning of its cDNA sequence for human (Wada et al. 1999) bovine (Miyagi et al. 1999) and mouse (Hasegawa et al. 2000).

Neu3 and gangliosides co-localize in Triton X-100-insoluble lipid rafts (Kalka et al. 2001). The nonrandom distribution of Neu3 at the cellular surface introduces the possibility that the biological effects of this enzyme might be due to the local reorganization of GSL-based signaling units. Remarkably, Neu3 modulates cell surface GSL composition by *trans* interactions, hydrolyzing substrates on the surface of neighboring cells (Papini et al. 2004).

In colon and renal cancer, this sialidase appears to be responsible for acting on GM3, thereby maintaining high cellular levels of lactosylceramide that can exert a Bcl-2-dependent antiapoptotic effect, contributing to survival of the cancer cells and subsequent tumor progression (Kakugawa et al. 2002; Ueno et al. 2006).

Neu3, together with plasma membrane-associated β-galactosidase and β-glucosidase, can act on gangliosides to produce bioactive ceramide at the cell surface of cultured human fibroblasts (Valaperta et al. 2006). The structure of plasma membrane-associated ß-galactosidase is still unknown; it could be the lysosomal enzyme, transferred to the plasma membrane by the fusion of lysosomes with the plasma membrane. Two distinct β-glucosidases are associated with the plasma membranes of fibroblasts. One is the lysosomal enzyme GBA1; the other, GBA2, displays a different structure. Expression of either Neu3, β-galactosidase, or β-glucosidase can affect the expression/activity of the others. Overexpression of Neu3 resulted in increased expression/activity of β-galactosidase and β-glucosidase. This resulted in an increase in ceramide and a shift from cell proliferation to cell death by apoptosis. Nevertheless the increase of cell surface ceramide was paralleled by a very scant reduction in ganglioside GM3, a substrate for Neu3, and the main ganglioside in fibroblasts. In fact, overexpression of Neu3 led to increased expression of GM3 synthase. This may be how cells avoid reduction of GM3 by the concomitant increase in Neu3, β-galactosidase, and β-glucosidase activities that combined catalyze conversion of GM3 to ceramide. The increased expression of sialyltransferase 1 (SAT1) increases its action on lactosylceramide depleting its availability for the biosynthesis of globotrihexosylceramide. Figure 10.4 shows relationships between several enzymes of GSL metabolism. As for Neu3, plasma membrane β-galactosidase and β-glucosidase display *trans* activity in living cells by acting on substrates on neighboring membranes in the absence of activator proteins or detergents (Papini et al. 2004; Aureli et al. 2009).

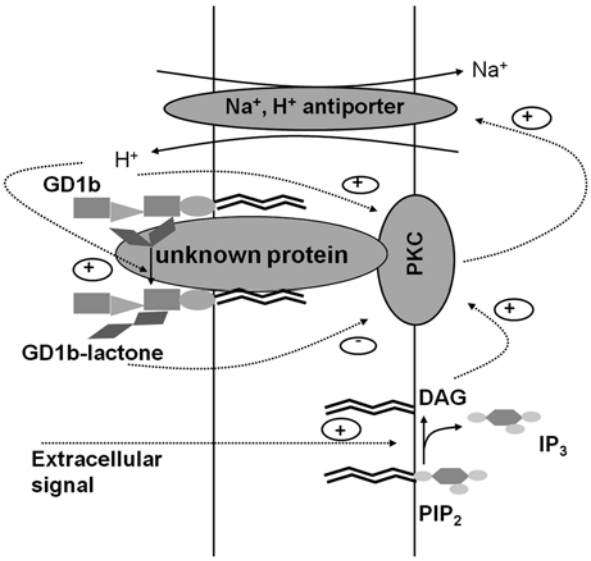


**Fig. 10.5** Monolactone formation due to the acidic plasma membrane microenvironment. The catalytic protons are generated by the sodium/proton pump located at the cell surface and activated through the action of protein kinase C (PKC). The local acidic microenvironment is directly responsible for lactone formation

The presence of active β-hexosaminidase A in the external leaflet of the plasma membrane has also been found in cultured fibroblasts (Mencarelli et al. 2005). Immunological and biochemical characterization of the membrane-associated β-hexosaminidase indicated that this enzyme has the same structure as the lysosomal enzyme. This led to the hypothesis that a regulated fusion of lysosomes with the plasma membrane might be a general mechanism of repair for the plasma membrane (Reddy et al. 2001) and could also provide a way for lysosomal GSL-metabolizing enzymes to reach the cell surface where, together with specific and different membrane-associated enzymes, they could play an active role in remodeling the GSL composition of the plasma membrane.

Information is also available about in situ sialylation of cell surface gangliosides. The original report on the presence of a synaptosomal membrane sialyltransferase in calf brain (Preti et al. 1980) was confirmed by metabolic studies in chicken embryos (Matsui et al. 1986) and rat brains (Durrie et al. 1988; Durrie and Rosenberg 1989). More recently, it was shown that exposure to dexamethasone markedly increased GM3 synthesis, due to enhanced gene expression and increased enzyme activity of SAT1. Metabolic studies indicated that this event is localized at the plasma membrane (Iwamori and Iwamori 2005), thus confirming that glycolipid sialylation might occur outside the Golgi compartment, contributing to the local modulation of the cell surface GSL pattern.

A further possibility for modification of the oligosaccharide structure of gangliosides is by the lactonization of molecules containing a disialosyl residue, such as GD1b. Ganglioside lactones are present as minor components in vertebrate brains (Sonnino et al. 1983; Riboni et al. 1986). GD1b monolactone formation, in the presence of catalytic protons, has been studied in vitro (Bassi et al. 1989) (Fig. 10.5), and it has been shown that lactonization influences the conformation, aggregative

(Acquotti et al. 1987) and biological properties of GD1b (Bassi et al. 1991). GD1b is able to directly interact with several cellular proteins (Prinetti et al. 2000b) and to modulate several plasma membrane-associated protein kinase activities (Bassi et al. 1991), while GD1b-lactone does not or does so in a very reduced way (Bassi et al. 1991; Sonnino et al. 1990). This suggests that lactonization/delactonization might represent a localized event able to trigger specific ganglioside-mediated cellular events. In vivo GD1b lactonization occurs in neurons through a process that suggests the presence of a specific enzyme associated with the plasma membrane (Bassi et al. 1994).

## 10.5 Plasma Membrane Glycosphingolipid Hydrolases in the Nervous System

### *10.5.1 Sialidase Neu3*

The first plasma membrane-associated enzyme identified that was involved in ganglioside catabolism was the sialidase Neu3 (NEU3, EC 3.2.1.18). It can be considered an ubiquitous enzyme as it is expressed albeit at different levels in the plasma membranes of most normal and pathological human tissues such as the human brain (Kopitz et al. 1994), normal colon, as well as colon rectal carcinoma tissues, hepatic tumors, and kidney carcinomas (Monti et al. 2002; Miyagi et al. 2008b; Miyagi et al. 2008a; Ueno et al. 2006; Kakugawa et al. 2002). In addition, its expression and activity were also found in normal and pathological cell lines such as erythroid and erythroleukemic cells (Venerando et al. 2002; Tringali et al. 2007b; Tringali et al. 2007a), fibroblasts (Chigorno et al. 1986), neurons, neuroblastoma cells (Schengrund and Repman 1982), breast ductal cancer T47D cells, colon carcinoma CaCo2 cells, colorectal adenocarcinoma HT29 cells, different types of ovarian cancer cells and cervix adenocarcinoma HeLa cells (Kakugawa et al. 2002).

Neu3 catalyzes the hydrolysis of both α2–8 and α2–3 external ketosidic linkages but is ineffective on inner α2–3 sialyl residues. An increase in Neu3 activity modifies cell surface ganglioside composition by catalyzing the conversion of polysialylated species of Gg4 and Gg3 to monosialoderivatives and GM3 to lactosylceramide. These changes have significant effects on neuronal differentiation and apoptosis in both normal and pathological cells (Kakugawa et al. 2002; Valaperta et al. 2007; Valaperta et al. 2006). In mouse and human neuroblastoma cells, the pharmacologically induced neuronal differentiation is accompanied by an increase in Neu3 expression and activity (Proshin et al. 2002). Neurite outgrowth can also be induced by transfection of cells with Neu3. In addition, an increase in Neu3 activity enhanced extension and/or branching of neurites promoted by exposure of neuroblastoma cells to 5-bromodeoxyuridine (Hasegawa et al. 2000). Conversely, in SK-N-MC neuroblastoma cells, inhibition of Neu3 activity resulted in the loss of neuronal differentiation markers (Kopitz et al. 1994; von Reitzenstein et al. 2001).

In cultured rat granule cells, Neu3 increased during differentiation and remained constant during aging (Aureli et al. 2011c). In cultured hippocampal neurons, Neu3 activity regulated the plasma membrane content of GM1 and was essential for axonal growth and regeneration after axotomy (Rodriguez et al. 2001). In these neurons, Neu3 activity is asymmetrically concentrated at the end of a single neurite and determines its axonal fate by a local increase in TrkA activity (Da Silva et al. 2005). However, reduction of Neu3 expression did not prevent induction of neuroblastoma cell differentiation (Valaperta et al. 2006). The nonrandom distribution of Neu3 at the cell surface (Kalka et al. 2001) introduces the possibility that the biological effects of this enzyme might be due to local reorganization of GSL-based signaling units, not just on cells with which Neu3 is associated, but on neighboring cells as well, due to *trans* interactions (Papini et al. 2004).

Taking into account the high concentration of gangliosides in the nervous system, and the very high and progressive increase of Neu3 and total sialyltransferase during neuronal differentiation, it is possible to hypothesize that a sialylation–desialylation cycle on the plasma membrane has a specific role during neuronal cell specialization, especially during cell stages in which it is necessary to build specialized membranes such as axonal protrusion and elongation, dendritic arborization, and synaptogenesis. These enzymes could also be essential for axon repair and/or synaptic function.

### *10.5.2 β-Glucocerebrosidases*

At least three different β-glucocerebrosidases have been described: a β-glucocerebrosidase (GBA, GBA1, EC 3.2.1.45) sensitive to inhibition by conduritol B epoxide (CBE), associated primarily with lysosomes (Neufeld et al. 1996); a cytosolic β-glucosidase (GBA3, EC 3.2.1.21) (Daniels et al. 1981); and a non-lysosomal β-glucosylceramidase (GBA2, EC 3.2.1.45) (van Weely et al. 1993).

GBA2 is able to catalyze the hydrolysis of glucosylceramide both at the cell surface and in the endoplasmic reticulum (van Weely et al. 1993; Korschen et al. 2012). In fact, the cellular localization of GBA2 is still controversial, since it has been described as being associated with endosomal vesicles, the plasma membrane, and the endoplasmic reticulum. Analysis of data describing its localization indicates that it depends on the cell type and cell stage. Up to now, most of the information about GBA2 localization came from studies performed on cells overexpressing GBA2 as a fusion protein with green fluorescence protein (GFP); therefore, further investigation is needed to define its cellular topology. Database searching of GBA2 cDNA sequences revealed apparent orthologs of this enzyme in species ranging from Drosophila to Arabidopsis to vertebrates, indicating that the protein is highly conserved and suggesting its functional importance. Study of GBA2 expression and activity showed that in humans, this enzyme is abundant in the brain, heart, skeletal muscle, and kidney (Matern et al. 2001), whereas in mouse it is most abundant in the testis and brain (Yildiz et al. 2006).

A recent study using GBA2 knockout (KO) mice showed an abnormal accumulation of glucosylceramide in multiple tissues, including the brain, liver, and testis. The KO mice had normal bile acid metabolism and, apparently, no impairment in the CNS; however, the accumulated glucosylceramide led to decreased fertility due to formation of misshapen spermatozoa (Yildiz et al. 2006). More recent studies indicated that mutations in GBA2 caused autosomal recessive cerebellar ataxia with spasticity in humans (Martin et al. 2013; Hammer et al. 2013). Moreover, antisense morpholino oligonucleotides targeting the GBA2 orthologous gene in zebrafish led to abnormal motor behavior and axonal shortening/branching of motoneurons. This condition could be rescued by transfection with human wild-type mRNA for GBA2 but not with the mRNA containing the missense mutation found in GBA2 in patients affected by autosomal recessive cerebellar ataxia with spasticity. These data suggest a specific role for GBA2 in the control of the cellular glucosylceramide–ceramide balance that could be responsible for the onset of motoneuron defects (Martin et al. 2013). As mentioned before, homozygous GBA2 knockout mice showed no apparent neurological signs, liver dysfunction, or reduced viability when observed at 4 months of age even when an accumulation of glycolipid species was observed by mass spectrometry in the brain, liver, and testis (Yildiz et al. 2006). Longer times may be required to observe a neurological phenotype in these mice, as was found in other mouse models of hereditary spastic paraplegia. The latter may reflect the fact that the neurological signs are very subtle during the first months of life (Ferreirinha et al. 2004; Soderblom et al. 2010). Impairment of their nervous system could also be obscured by the different structure of mouse corticospinal tracts compared to humans or zebrafish or by a compensation for the loss in GBA2 activity by other GBA enzymes during early stages of development (Martin et al. 2013). It should be noted that the GBA2 knockout mice lacked only exons 5 to 10 and retained 50 % of normal glucosidase activity. On this basis it has been hypothesized that accumulation of glucosylceramide in the ER and/or plasma membrane did not reach the threshold needed to cause neurological symptoms.

It has also been shown that GBA2 activity increases more than threefold during neuronal differentiation (Aureli et al. 2011a). β-Glucosidase activity also increases during differentiation of murine neuronal stem cells (Aureli et al. 2011a). However, in primary neuronal cultures, cell surface β-glucosidase activity is due primarily to the GBA2 enzyme whose activity increases more than threefold during neuronal differentiation. While the CBE-sensitive β-glucosidase enzyme contributes to the total plasma membrane (PM) β-glucosidase activity, the CBE-sensitive β-glucosidase/GBA2 ratio is 0.4 in the first stage of neuronal differentiation and drops to 0.25 in fully differentiated neurons (Aureli et al. 2011c). Conversely, in murine neuronal stem cells, plasma membrane β-glucosidase activity was largely due to the CBE-sensitive β-glucosidase enzyme, with the CBE-sensitive β-glucosidase/GBA2 ratio 0.75 in precursors and 2.3 in differentiated cells. The different behaviors of these enzymes in the two different cellular models could reflect the fact that, as a result of serum-induced murine neuronal stem cell differentiation, the neuronal component in the differentiated cells accounted for only about 10 % of the cells, with about 70–80 % by glial cells, whose contribution in terms of the two

different β-glucosidase activities could be very different than that of neurons. In fact, in nonneuronal cell lines such as human fibroblasts, we observed a ratio between CBE-sensitive β-glucosidase and GBA2 that was around 7 (Aureli et al. 2009). The lack of data on the activity of PM-associated glycohydrolases in cultures of astrocytes and oligodendrocytes indicates that future studies need to be done to assess this issue. Interestingly in studies of fibroblasts derived from patients affected by different variants of Gaucher disease (GD), the reduction in total GBA1 activity was paralleled by an increase in GBA2 activity and expression. This was particularly evident in fibroblasts from patients affected by type 2 GD (GD2), the most severe form of the neuronopathic type of GD (Aureli et al. 2012). Despite all of these observations, the link between GBA2 and neuronal differentiation as well as neurodegeneration is still unclear and merits further study.

Several years ago the presence of a cell surface CBE-sensitive β-glucosidase activity was described (Aureli et al. 2009). Studies of human fibroblasts derived from patients affected by GD showed that this plasma membrane-associated activity was significantly reduced with respect to fibroblasts from healthy patients permitting one to ascribe this activity to a deficiency in the GBA1 enzyme (Aureli et al. 2012).

Increased GBA1 and GBA2 activities on the plasma membrane of human fibroblasts and concomitant increase in ceramide are responsible for cell cycle arrest and apoptosis (Valaperta et al. 2006). This activity is due to both enzymes (Aureli et al. 2009).

A recent multicentre study demonstrated that mutations of GBA1 represent, to date, the most common genetic risk factor for Parkinson's disease (PD) (Sidransky et al. 2009). Importantly, a recent paper demonstrated that in neurons and brains from this type of PD patients, the lysosomal accumulation of glucosylceramide, the substrate for GBA1, directly influenced the abnormal lysosomal storage of α-synuclein oligomers resulting in a further inhibition of GBA1 activity (Mazzulli et al. 2011). These findings suggest for the first time that the bidirectional effect of decreased GBA1 activity and α-synuclein accumulation forms a positive feedback loop that may lead to self-propagating disease (Mazzulli et al. 2011). Up to now this process has just been described for the lysosomal function of GBA1, but due to its involvement in the production of apoptotic ceramide at the PM level, GBA1 associated with the cell PM could contribute to neuronal impairment in neurodegenerative diseases.

### *10.5.3 β-Galactosidases*

Two different β-galactosidases involved in GSL metabolism have been described: β-galactocerebrosidase (β-Gal-ase, GALC EC 3.2.1.46), which catalyzes the hydrolysis of galactose from galactosylceramide, lactosylceramide, and galactosylsphingosine, and the β-galactosidase (GAL, EC 3.2.1.23), which catalyzes hydrolysis of the terminal galactose of GM1 (Li and Li 1999). As is well known, loss of function

of β-galactosidase EC 3.2.1.46 is responsible for the disease globoid leukodystrophy (GLD, Krabbe disease), while deficiency of β-galactosidase EC 3.2.1.23 is the cause of GM1 gangliosidosis (Xu et al. 2010). Both these sphingolipidoses are characterized by an impairment of the CNS although the molecular bases are unclear.

In addition to the lysosomal enzymes, PM-associated β-galactosidase activity has been found in several cell lines (Aureli et al. 2011b). The identity of the protein, or proteins, responsible for the β-galactosidase activity present at the cell surface is still unknown. However, in living human fibroblasts, the presence of a β-galactosidase which displays a *trans* activity (on substrates belonging to the cell surface of neighboring cells) has been verified. It is active in the absence of detergents or activator proteins, suggesting that on the cell surface there is at least one enzyme with a β-galactocerebrosidase-like activity (Aureli et al. 2009). Using the same cells, it was shown that its expression is up-regulated by Neu3 overexpression and correlated with the onset of ceramide-mediated apoptosis (Valaperta et al. 2006). β-Galactosidase activity was measured during neuronal cell differentiation and aging, in both the total cell lysate and the plasma membrane fraction from rat cerebellar granule cells. Both activities were up-regulated during cell differentiation. As expected, β-galactosidase activity associated with the plasma membranes was much less than that found in the total cell homogenate. Total cell activity remained constant during differentiation and then increased fourfold during aging. In contrast, cell surface activity increased tenfold during differentiation and then doubled during neuronal senescence (Aureli et al. 2011c). Similar behavior was described for PM-associated β-galactosidase activity during neuronal differentiation of NSCs (Aureli et al. 2011a). β-Galactosidase activity has been proposed as a marker for senescence (Coates 2002; Dimri et al. 1995; Severino et al. 2000; Geng et al. 2010). The behavior of the PM-associated enzyme in rat cerebellar granule cells suggests that β-galactosidase activity could be used as hallmark of both neuronal differentiation and aging as well as of apoptosis in fibroblasts. Little is known regarding the functional role of PM-associated β-galactosidases. It has been hypothesized that they may act as cell surface receptors mediating various cell–cell and cell–matrix interactions responsible for cell migration, differentiation, and axonal protrusion (Evans et al. 1993; Huang et al. 1995). No data are available on their enzymatic properties.

### *10.5.4 β-Hexosaminidases*

β-Hexosaminidase is a dimeric enzyme that exists in three different isoforms. There are two different subunits for β-hexosaminidase, α (528 residues) and β (556 residues), encoded by two different but evolutionarily related genes, HEX A and HEX B (Triggs-Raine et al. 2001). Each subunit has its own active site: the β-subunit hydrolyzes uncharged substrates, whereas the α-subunit catalyzes cleavage of GalNAc from negatively charged ones (Bearpark and Stirling 1978; Kytzia and

Sandhoff 1985). However, dimerization is necessary for the enzymes in order to become fully functional. Thus, the α- and β-subunits can form three different β-hexosaminidase isoenzymes: Hex A (αβ), Hex B (ββ), and Hex S (αα). Only the αβ heterodimer Hex A is able to remove β-linked nonreducing terminal GalNAc from ganglioside GM2 and GalNAc-GD1a, in the presence of the GM2 activator protein, a specific cofactor of Hex A (Kolter and Sandhoff 2006).

Genetic defects in either of the genes encoding the α- and β-subunits of Hex A or the GM2 activator protein can result in GM2 accumulation in neural tissue leading to one of the three forms of GM2-gangliosidosis: Tay–Sachs disease, due to defects in the α-subunit (TSD, OMIN 2728800); Sandhoff disease, characterized by defects in the β-subunit (SD, OMIN 268800); and the AB variant of Sandhoff disease (OMIN 272750), in which both the subunits are affected (Bateman et al. 2011). The massive neuronal accumulation of GM2 is accompanied by progressive neurological deterioration affecting motor, cerebral, and spinocerebellar functions.

The presence of active β-hexosaminidase A in the external leaflet of the plasma membrane was found in studies of cultured fibroblasts (Mencarelli et al. 2005). Immunological and biochemical characterization of the membrane-associated β-hexosaminidase indicated that the enzyme has the same structure as that in lysosomes. This suggests that a regulated fusion process between lysosomes and the plasma membrane might be responsible for transport of lysosomal enzymes to the cell surface where the enzymes could function in the remodeling of the glycolipid content and pattern on the external leaflet of the plasma membrane. During differentiation of murine neuronal stem cells, plasma membrane β-hexosaminidase increased its activity, reaching a maximum in fully differentiated cells (Aureli et al. 2011a). On the other hand, analysis of the PM-associated glycohydrolase activity in fibroblasts derived from patients affected by different variants of Gaucher disease indicated that the PM-associated β-hexosaminidase increased only in cells derived from patients affected by the most severe neuronopathic form of GD (GD2) (Aureli et al. 2012).

Analysis of epithelial cells indicated the presence on the cell surface of an UDP-GalNAc: LacCer/GM3/GD3 *N*-acetylgalactosaminyl transferase able to act on exogenous GM3 (Crespo et al. 2010). Currently, no data are available regarding its activity on endogenously synthesized GSLs. However, its ability to act on exogenous substrates could be important in the plasma membrane remodeling process. In fact, the glycolipid composition of the plasma membrane could also be remodeled by the uptake of glycolipids from the extracellular environment (e.g., from other cells, lipoproteins, or molecules shed by other cells). These compounds, depending on the "cellular request" and on their aggregation, could be endocytosed or become components of the cell surface directly or after modification by the action of PM-associated enzymes. The coexistence of β-hexosaminidase and β-hexosaminyl transferase activity on the cell surface supports the hypothesis that there is a glycolipid cycle occurring on the plasma membrane that can have important biophysical effects on the membrane itself and affect events that regulate the "cell social life."

## 10.6 Conclusions

During neuronal development dramatic changes occur in GSL content along with a parallel reorganization of PM lipid domains enriched in GSLs (Yu 1994; Prinetti et al. 2001; Yu et al. 2004). While the driving forces guiding these modifications are not completely understood, some information regarding the possibility of fine-tuning the cell PM GSL composition has been obtained regarding a synergy in activity by different plasma membrane glycohydrolases (Valaperta et al. 2006; Aureli et al. 2009).

In rat cerebellar granule cells, increases in total cell ceramide content (eightfold from the 2nd to the 17th day in culture) and in that belonging to the sphingolipid-enriched domains (tenfold from the 2nd to the 17th day in culture) were observed. A parallel reduction in the endogenous content of both sphingomyelin and gangliosides was observed in sphingolipid-enriched domains of senescent cells relative to fully differentiated neurons (Prinetti et al. 2000a). The increase of ceramide could be explained by the well-known ceramide–sphingomyelin cycle that is known to correlate with apoptotic phenomena (Venable et al. 1995). The increased activity of the PM-associated glycohydrolases during cell aging supports speculation that the augmented ceramide in the PM could be derived from cell surface catabolism of the glycosphingolipids, as found in human fibroblasts (Valaperta et al. 2006). As reported (Rodriguez et al. 2001; Da Silva et al. 2005), the sialidase Neu3 is able to influence extension and symmetry of axons in neuronal cells, possibly by inducing a local change in PM sphingolipid composition at the axonal cones. All these data support the idea that modulation of the activities of other PM-associated glycohydrolases during neuronal differentiation could affect differentiation itself and could also help define the curvature properties of specific areas of the PM (such as synapses or the negative curvature of the membrane near the axon protrusion) by a rapid in situ modification of GSL components. An example of the latter is provided by the geometry of synapses. Typically it is characterized by a succession of PM regions with negative or positive curvatures that correlate respectively to an enrichment of the area with simple sphingolipids or the enrichment of more complex GSLs (Sonnino et al. 1994; Brocca and Sonnino 1997). On the other hand, an aberrant increase in cell surface glycohydrolases can increase production of apoptotic ceramide and lead to the onset of neuronal impairment. For these reasons, the balance between glycosylation and de-glycosylation events at the cell surface could be a very important mechanism for maintaining the appropriate neuronal physiology.

**Conflict of Interest** All the authors declare that they have no conflict of interest.

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