UNIVERSITA' DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE BIOCHIMICHE, NUTRIZIONALI E METABOLICHE

DOTTORATO DI RICERCA IN BIOCHIMICA
CICLO XXIII
BIO/10

TESI DI DOTTORATO DI RICERCA

PLASMA MEMBRANE ASSOCIATED GLYCOHYDROLASES: KINETIC PARAMETERS CHARACTERIZATION AND BIOLOGICAL FUNCTION.

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Anno Accademico 2009-2010

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ABBREVIATIONS

AMP-DNM	*N*-(5-adamantane-1-yl-methoxy)pentyldeoxynojirimycin
BME	Basal Modified Eagle's Mediumù
CBE	Conduritol B Epoxide
Cer	Ceramide
DMEM	Dulbecco Modified Eagle's Medium
EIPA	5-(N-Ethyl-N-isopropyl)amiloride
FBS	Fetal bovine serum
GBA2	Non-lysosomal β-glucosylceramidase
GlcCer	Glucosyl ceramide
GSL	Glycosphingolipid
Hex	Hexosaminidase
HMU-PC	6-Hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine
HPTLC	High performance thin layer chromatography
LacCer	Lactosyl ceramide
LacCer	Lactosylceramide
MEM	Minimum Essential Medium with Earle's Salt
MUB	4-Methylumbelliferone
MUB-Gal	4-Methylumbelliferyl-β-D-galactopyranoside
MUB-Glc	4-Methylumbelliferyl-β-D-glucopyranoside
MUG	4-Methylumbelliferyl-β-N-acetylglucosaminide
MUGS	4- Methylumbelliferyl-β-N-acetylglucosaminide-6-sulphate
Neu3	PM associated sialidase

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PM	Plasma membrane
PVDF	Polyvinylidene di fluoride
SM	Sphingomyelin
SMase	Sphingomyelinase
ST-1	GM3 synthase, CMP-NeuAc: LacCer α2-3 sialyltransferase
ST-II	GD3 synthase, CMP-NeuAc: GM3 α2-8 sialyltransferase
ST-IV	CMPNeuAc: GA1/GM1/GD1b/GT1c α2-3 sialyltransferase
β-Gal	β-Galactosidase
β-GlC	β-Glucosidase

Ganglioside nomenclature is in accordance with IUPAC-IUBMB recommendations (IUPAC-IUBMB 1998)

ABSTRACT

Abstract

Plasma membrane (PM) sphingolipid pattern and content are the result of a complex network of metabolic pathways, including those potentially involving the activity of PM sphingolipid hydrolases and sphingolipid synthases (*1, 2*). In human fibroblasts it has been observed a crosstalk between the expression and the activity of membrane associated glycohydrolases (*3*), furthermore, qualitative and quantitative changes in glycosphingolipids, together with changes in the expression of the corresponding glycosyltransferases, have been reported along neuronal differentiation and aging (*2, 4-8*).

Using human fibroblasts we set up an HTA (high throughput assay) method able to measure the activity of β-galactosidase, CBE sensitive β-glucosidase, GBA2 β-glucosidase, SMase and β-hexosaminidases, enzymes working directly at the plasma membrane level using MUB derivates as substrate. Afterwards we applied this assays in order to study these PM associated activities in rat cerebellar granule cells along differentiation and aging in culture.

We started to characterize the kinetic parameters of these enzymes working directly on the external leaflet of the PM in living cells: first of all, we performed an assay using cell cultured medium at different pH. Our data showed higher activity of CBE sensitive β-glucosidase at pH 6.2, GBA2 β-glucosidase at pH 5.7, β-hexosaminidase at pH 5.10 while β-galactosidase, SMase and β-hexosaminidase A at pH 5.45. We could distinguish between CBE sensitive β-glucosidase and GBA2 β-glucosidase using specific inhibitors that are CBE and AMP-DNM respectively, therefore we determined Km and Vmax of CBE sensitive β-glucosidase and GBA2. Thought the pH conditions are not those typically associated with the PM, at least 7 different families of transporters/exchangers are present at this level and are capable of varying the pH locally. In order to verify whether the varying of the membrane potential acting on these pumps can influence also the activity of these enzymes, we carried out preliminary experiments using EIPA, an inhibitor of NHEs (Na+/H+) exchangers. What we achieve is that exists a correlation between the action of the pump and the enzymes. So, all our data confirm the presence of this PM associated activities. This could play an important role in the cell social life and in particular our data show an involvement of sphingolipid hydrolases during the neuronal differentiation.

INTRODUCTION

PLASMA MEMBRANES

The surface of eukaryotic cells is a complex assembly of a variety of molecular components which actively partitions the interior of the cell from the external environment. Plasma membrane is composed of a double layer of lipids where many diverse proteins are embedded while others are simply associated by lipid anchors. The lipid composition of the plasma membrane (Figure 1) is extremely complex, consisting of an incredible list of different lipid species, classified according to head-group and backbone structures. These include glycerophospholipids, sphingolipids and cholesterol (*9, 10*).

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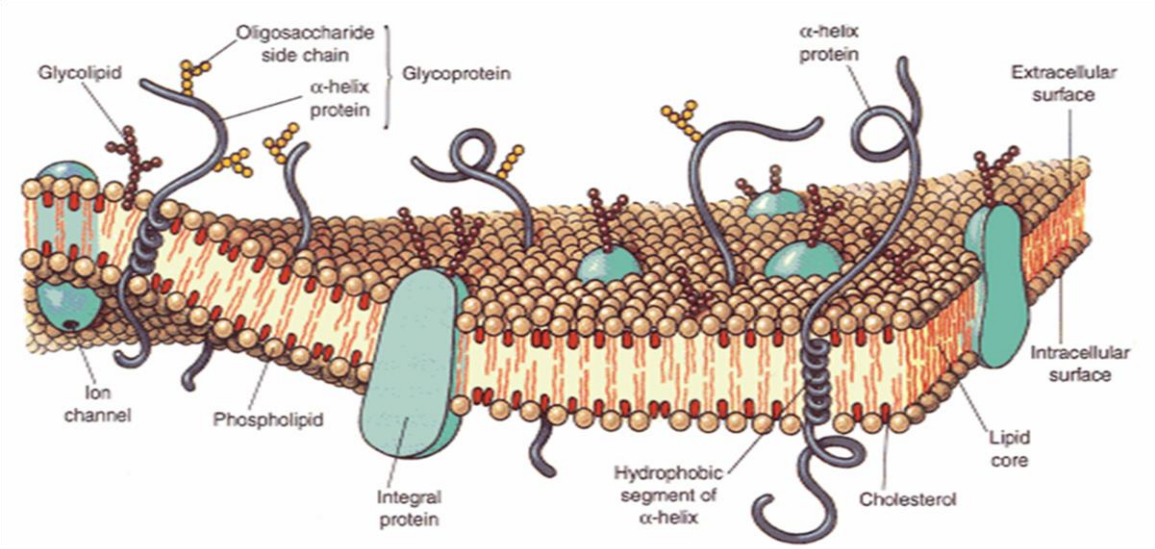


Fig. 1: plasma membrane

The eukaryotic cell is functionally compartmentalized via membrane-limited organelles that continually exchange biomolecules by a variety of membrane trafficking mechanisms (*11, 12*). In the face of this dynamic exchange, heterogeneity in the lipid composition of the membranes of different organelles appears to be maintained. For instance, the plasma membrane is highly enriched in cholesterol and glycosphingolipids, while the endoplasmic reticulum (ER) is poor in these components (*13, 14*). In addition, there is a transbilayer lipid compositional asymmetry within the same membrane (*9*) that could have important functional consequences. A dramatic demonstration is represented by what occurs during apoptosis when the predominantly inner-leaflet lipid species, phosphatidylserine, fails to be

actively 'flipped' and accumulates at the outer leaflet, thus serving as a signal for clearance of apoptotic cells by macrophages with scavenger functions (*15, 16*). Although precise details of how compositional heterogeneity is achieved are not available, it is abundantly clear that this is done by a complex mechanism involving lipid synthesis, turnover and active transport.
A great contribute to determine the high complexity of the plasma membrane organization and of its function is also given by the protein components. Plasma membrane proteins function in several different ways: many of them play a role in the selective transport of certain substances across the phospholipid bilayer, either acting as channels or active transport molecules. Others function as receptors, which bind information-providing molecules, such as hormones, and transmit corresponding signals based on the obtained information to the interior of the cell. Membrane proteins may also exhibit enzymatic activity, catalyzing various reactions related to the plasma membrane.

MEMBRANE LIPIDS

The two highly heterogeneous groups of glycerophospholipids and of sphingolipids are, together with cholesterol, the complex lipid components of cell membranes.

GLYCEROPHOSPHOLIPIDS

Glycerophospholipids are glycerol-based phospholipids and they are the main lipids of eukaryotic cell membranes. They consists of a small polar head group (at the inner and outer surfaces of the cell membrane) and two long hydrophobic chains (pointing to each other and forming a fatty, hydrophobic centre).

SPHINGOLIPIDS

Sphingolipids are minor cell components, residing in the external layer of the plasma membrane (*17*) with the hydrophilic head group protruding toward the extracellular environment. Many reports indicated multiple functional roles for membrane sphingolipids, including the modulation of the properties of important membrane enzymes (*18-22*). The lipid moiety of sphingolipids is named ceramide (Figure 2). Ceramide is the most simple sphingolipid and it is constituted by a long chain amino alcohol, 2-amino-1,3-dihydroxy-

octadec-4-ene, whose trivial name is sphingosine (Figure 3) connected to a fatty acid by an amide linkage. Of the four possible configurations of sphingosine, only the 2*S*,3*R* is present in nature (*23, 24*).

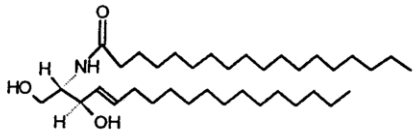


Fig. 2: ceramide

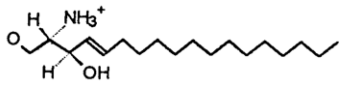


Fig. 3: sphingosine

Ceramide is the common precursor of complex sphingolipids, which are synthesized by addition of polar molecules to hydroxyl group in position 1 of the sphingoid base (*25*). The different classes of sphingolipids are characterized by different polar groups: phosphocholine is found in sphingomyelin (SM), monosaccharides in cerebrosides, saccharidic chains in complex glycolipids. Gangliosides are a peculiar class of acid glycolipids, that get their acidity from sialic acid (*26*).

The glycolipids are clearly significant contributors to the structure of the outer leaflet of most eukaryotic cell membranes. The molar ratio of these molecules relative to the other major membrane lipids (phospholipids, cholesterol, and glycerolipids) varies from being rather minor (e.g. <5% in erythrocytes) to being a very major component (e.g., 30% of the total lipids in neuronal plasma membranes). Although glycolipids are found throughout all eukaryotes, the nature of the core structures can vary in different taxa. Ceramide, and inositolphosphate-amides are major sphingolipids in fungi and plants. Variations in type, number, linkage and further modification of sugar and sialic acid residues within the oligosaccharide chain but also the different composition of the lipid moiety give rise to a combinatorial variety (*27*) of naturally occurring glycosphingolipids (GSLs).

They can be classified into series, which are characteristic for a group of evolutionarily related organisms. Apart from the species dependence, GSLs form cell-type specific patterns on the cell surface. In particular sialic acid containing GSLs of the ganglio-series, the gangliosides, are abundant on neuronal cells and contribute to the function of the nervous system. Cell surface glycolipid patterns change with cell growth, differentiation, viral transformation and oncogenesis (*28*). Cell-type-specific glycolipid expression and stable lipid

patterns indicate a tight regulation of their biosynthesis, degradation and intracellular transport (*29*).

In humans, inherited defects of GSL- and sphingolipid catabolism give rise to lysosomal storage diseases, the sphingolipidoses: the function of animal glycosphingolipids has been analysed in cells from human patients with defects in sphingolipid catabolism (*27*).

CHOLESTEROL

Cholesterol, which is the third main important constituent of cell membranes, has a rigid ring system and a short branched hydrocarbon tail. Cholesterol is largely hydrophobic but it has one polar group, a hydroxyl, making it amphipathic. Cholesterol is an important lipid in this context since it is known to regulate the function of neuronal receptors and thereby affecting neurotransmission. Although the brain is highly enriched in cholesterol, the organization and dynamics of brain cholesterol is still poorly understood. Brain cholesterol is synthesized *in situ* and is developmentally regulated. The organization, traffic, and dynamics of brain cholesterol are stringently controlled since the input of cholesterol into the central nervous system is almost exclusively from *in situ* synthesis as there is no evidence for the transfer of cholesterol from blood plasma to brain (*30*).

ROLE OF GLYCOSPHINGOLIPIDS

The vast range of head group structures found on the cell surface suggest that they play an important role in cell signalling and cell activation. In addition, the expression pattern of GSLs undergoes certain changes during development and neoplastic transformation, since GSLs act~~s~~ as a receptor sites in cell-cell recognition (figure 4). They form cell type specific profiles which characteristically change in development, differentiation and oncogenic transformation, suggesting their implication in fundamental cellular processes like:

- ✓ growth
- ✓ differentiation
- ✓ morphogenesis
- ✓ cell to matrix interaction
- ✓ cell to cell communication

There are many putative biological roles of glycosphingolipids, of which only a few have been conclusively established (Figure 4).

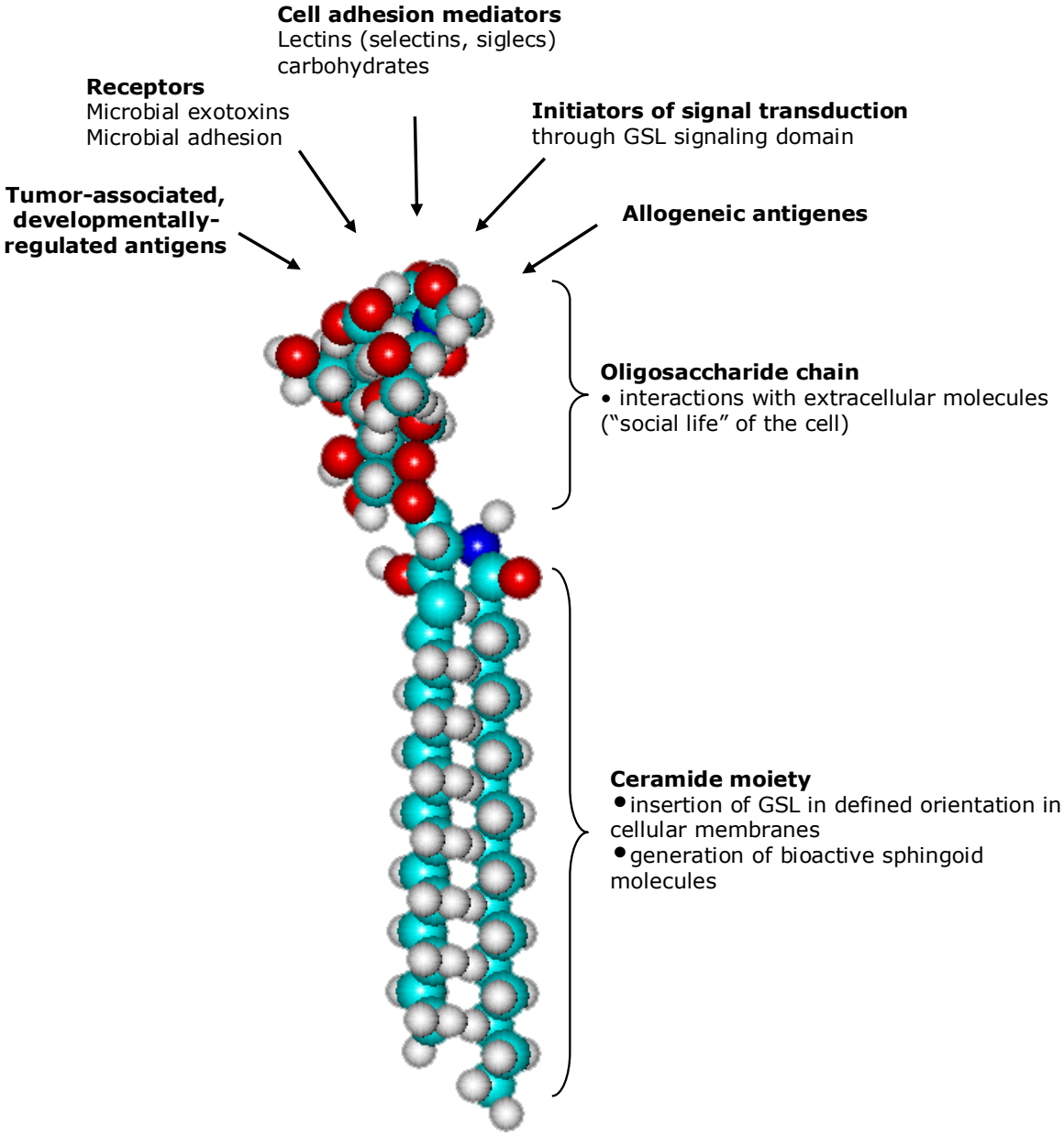


Fig. 4: sphingolipids functions

From a physical point of view, glycosphingolipids may have an organizing role in the cell membrane. For example, glucosylceramides (GlcCer) and ceramides are thought to be critical components of the epidermal permeability barrier. In this regard, it is noteworthy that they are similar to glycophospholipid (GPI) anchors having long acyl chains (24–26 carbons). It is thought that this common structural feature allows both types of lipids to

extend into the inner leaflet of the phospholipid bilayer of cell membranes and also causes them to associate with each other and with cholesterol, forming putative “rafts” in the micromembrane (some investigators report the existence of distinct glycosphingolipid-enriched domains free of cholesterol that are thought to be involved in signal transduction via cytosolic proteins) (*31*). Such aggregations of glycosphingolipids and GPI-anchored proteins are thought to form in the *trans*-Golgi region and then get targeted to the apical domains of polarized epithelial cells that line the lumen of various body cavities. Here, they can represent as much as 50% of the total lipid concentration of the outer leaflet of the apical membrane, likely providing physical protection against the hostile environment often encountered in such lumens, as well as specific binding sites for the adhesion of symbiotic bacteria. In the same location, other structural aspects of the same molecule can be utilized as highly specific receptor targets for a variety of bacteria, toxins, and viruses. The classic and well-established finding is that the β subunit of cholera toxin binds to the ganglioside GM1, triggering a conformational change and delivering the toxic α subunit to the interior of the cell.

A recurring theme in the literature is the specific physical association of certain glycosphingolipids with certain cell membrane receptors. Although the physical mechanism of such interactions is not certain, there is considerable evidence indicating that they can alter the biology of these receptors. For example, the tyrosine phosphorylation of the epidermal growth factor (EGF) receptor is specifically down-regulated by adding the ganglioside GM3, whereas the closely related structure sialylparagloboside has a similar effect only on the insulin receptor. A small modification of the GM3 molecule (de-N-acetylation) results in exactly the opposite response (stimulation of EGF receptor tyrosine phosphorylation). Related studies demonstrate the opposing effects of GM3 and LacCer on cell growth. However, many of these effects are demonstrated following the exogenous addition of glycosphingolipids to cells. Thus, although at least a portion of the added glycosphingolipids are incorporated into the membrane, one cannot rule out other effects caused by the micellar forms of the added glycosphingolipids that can also be bound to cell surface proteins. In balance, the stereospecificity of these effects for the fine structure of the glycan portion of the glycosphingolipids implies that defined mechanisms must be involved.

Similar findings have been reported for the specific effects of ganglioside GQ1b in inducing neuritogenesis, and a suggestion has been made that this involves the NGF receptor and/or an ectoprotein kinase. Again, final mechanistic conclusions are lacking. Regardless, these findings have encouraged other investigators to try ganglioside infusions in a variety of central nervous system disorders, including stroke and demyelinating disorders. Particularly in stroke, there are several reports of beneficial effects of infusing gangliosides into the bloodstream of patients. Likewise, direct topical applications of conduritol B epoxide, a specific irreversible inhibitor of β-glucocerebrosidase, increased GlcCer levels in the basal, proliferative cell layer (four fold increase) and stimulated proliferation. Simultaneous treatment with conduritol B epoxide plus GlcCer resulted in an additive increase in DNA synthesis, strongly suggesting that GlcCer directly stimulates epidermal mitogenesis.

It has also been suggested that gangliosides are involved in thermal adaptation of neuronal membranes. The data suggest a general rule that "the lower the environmental temperature the more polar is the composition of brain gangliosides." Studies with model bilayer membranes also indicate that gangliosides can modulate basic membrane properties in a thermosensitive manner.

In polarized cells such as epithelia, most GSLs are enriched in the apical, rather than the basolateral, surface (*32, 33*). GSLs are known to be of vital structural and functional importance to cells and to be extensively involved in various aspects of cell regulation. They participate in cell differentiation and intercellular communication events, for example, by controlling cell adhesion, mediate diverse functions of the immune system, and modulate membrane-bound enzymes and receptors (*34*). Specific gangliosides were identified as binding sites for toxins (*35*), viruses (*36*) and bacteria (*37*), and as co-receptors for hormones and growth factors (*36*). Several lines of evidence indicate that gangliosides contribute vitally to the function of the nervous system, for example, as modulators of neuronal calcium or neuronal differentiation and development (*38*). Several lipophilic products of GSL metabolism such as sphingosine and ceramide were found to function as signal transducing molecoles (*39*). Furthermore, others results suggest that GSLs are involved in the formation of rafts and caveolae and may thereby contribute to various signal transduction processes (*40*). In fact GSLs are not randomly distributed on the plasma membrane, but rather concentrated in restricted membrane areas (*41, 42*) due to their spontaneous segregation

respect to glycerophospholipid: it can be predicted that their local concentration in specific "lipid membrane domains" is very high.

SPHINGOLIPIDS AND NEURONS

Remarkably, membrane segregation of SL seems to be higher in neurons (*43-46*) than in any other cell type so far investigated. The presence of (glyco)sphingolipids deeply affects the structural properties of the cellular membrane. GSLs included in PC bilayers imply a curvature stress to the membrane that is probably relevant in the stabilization of the architecture of polarized cell membrane areas (such as the pre- and postsynaptic areas in neurons) and for the membrane geometry dynamics in processes such as vesiculation and budding. For this reasons the understanding of the metabolic regulation and the roles of sphingolipids is a major challenge, and particular attention has been paid to determining the roles of sphingolipids in neuronal development and in the central nervous system (CNS).

It is possible to observe drastic and consistent changes in ganglioside expression during neuronal development, particularly during the earliest stages. Many studies have shown that the qualitative and quantitative changes in ganglioside expression in the nervous system correlate with certain cellular events during development.

These observations, followed by several report, indicate the vital importance of glycosphingolipids in the "cell social life" and in particular regarding the relationship between GSL expression and cellular events in the development of the vertebrate nervous system, including that of humans (*4-8*). Available evidence indicates that GSLs have specific functional roles essential for survival, proliferation and differentiation during brain development including control of neuritogenesis (*22, 47, 48*), modulation of receptor and proteins and synaptic transmission (*49*) and cell-to-matrix interactions (*50*).

The crucial role of GSL in the development and maintenance of the proper functions of nervous system has been demonstrated by different evidences:

- GSL patterns undergo deep qualitative and quantitative modifications during the development of the nervous system: in chicken (*51*), rodent (*52*), and human brain (*4*), the total gangliosides content increases a lot in the postnatal life compared to the embryonic stages. These increases were accompanied by a dramatic shift from simple gangliosides (GM3 and GD3) to more complex species (GM1, GD1a, GD1b, GT1b). A

similar increase in the amount and in the complexity of gangliosides has also been observed during differentiation in cultured neurons of different origin and in mouse neural precursor cells (*51-60*). In humans, the phase of rapid ganglioside increase started from the sixth month of gestation reaching the maximum value at about 5 years of age. Along the adult life, a progressive loss of gangliosides with aging has been reported in human and mouse brain. During mild-embryonic stages of mouse brain development, GlcCer, but not GalCer or sulfatide, is expressed (*52*). Their synthesis starts in the embryonic development when oligodendrocytes enter terminal differentiation and is upregulated during the postnatal extension of the myelin sheaths (*61*).

- Experimental manipulations allowing modification of the concentration or pattern of GSL in the plasma membrane profoundly affect the behavior of neural cells. The addition of exogenous gangliosides exerts neuritogenic, neurotrophic, and neuroprotective effects in cultured neurons and neural cell lines and in animal models of neural lesions (*22, 62-65*). In particular, GM1 ganglioside is able to potentiate the neuritogenic effect of nerve growth factor (NGF) in PC12 cells, i.e., it is able to induce neuronal differentiation in the presence of an NGF concentration that is ineffective by itself (*66-68*). Increased surface expression of GM1, by treatment cells with bacterial sialidase, potentiated PGE1-induced neurite formation (*69, 70*). Furthermore, administration of exogenous GM1 and GM3 induced c-Src activation and neuritogenesis in neuroblastoma cells (*71*). Treatment with pharmacological inhibitors of ceramide synthase or ceramide glucosyltranferase, or selective depletion of cell surface SL, achieved by treating living cells with bacterial sphingomyelinases (*72, 73*)or with endoglycoceramidase (able to remove the oligosaccharide chain from cell surface GSL) (*74*), caused SL depletion and disorganization of SL-enriched domains (*75-79*), thus affecting domain-mediated biological functions, including survival in neurotumoral cell lines and oligodendrocytes, axonal transport and sorting (*80-83*), and finally TAG-1 signaling in cerebellar neurons (*74*).
- Many pieces of evidence indicated that SL biosynthesis is necessary for nervous system development. Blockade of (glyco)sphingolipid biosynthesis by pharmacological inhibition of GlcCer synthase or ceramide synthase reduced axonal elongation, branching synapses formation and activity (*84*) in cultured hippocampal and

neocortical neurons (*85, 86*), behind to this a pharmacologically inhibition of the β-glucocerebrosidase with CBE caused an increase in the number of axonal branches and a corresponding increase in the length of the axon plexus. Neural cell-specific deletion of GlcCer synthase in mice led to severe neurological defects in the early postnatal life and death within 3 weeks (*87*), demonstrating the importance of GSL for the maturation of the nervous system. On the other hand, pharmacologically induced stimulation of GSL biosynthesis stimulated neurite outgrowth, formation of functional synapses and synaptic activity in cultured cortical neurons (*84, 88*), and the induced expression of GD3 synthase was able to switch neuroblastoma cells to a differentiated phenotype (*89*). NGF- and forskolin-induced neuronal differentiation in PC12 cells was accompanied by the up-regulation of several glycosyltransferase activities (GalGb3-, GM3-, GD1a- and GM2 synthases) (*90*), and bFGF-stimulated axonal growth in cultured hippocampal neurons resulted in the activation of ceramide glucosyltranferase (*91*). It has been recently suggested that the activity of the plasma membrane-associated ganglioside sialidase Neu3 might have a role in modifying the cell surface ganglioside composition, causing a decrease of GM3 and shift from polysialylated ganglioside species to GM1, with deep consequences on very important cellular events, including neuronal differentiation. In neuroblastoma cell lines, Neu3 expression increased during pharmacologically induced neuronal differentiation, and Neu3 gene transfection induced neurite outgrowth (*92*) and enhanced the effect of differentiating agents on the extension or branching of neurites (*93*). Conversely, inhibition of plasma membrane sialidase activity resulted in the loss of neuronal differentiation markers (*94-96*). In cultured hippocampal neurons, Neu3 activity regulated the local GM1 concentration, determining the neurite's axonal fate by a local increase in TrkA activity (*97*) and affecting axonal regeneration after axotomy (*98*).

It was recently reported that the sialidase Neu4 was dominantly expressed in the mouse brain (*99-101*) and its expression was relatively low in the embryonic stage and then rapidly increased at 3-14 days after birth. Moreover during retinoic acid-induced differentiation, Neu4 expression was down-regulated in Neuro2a cells and the overexpression of Neu4 resulted in suppression of neurite formation; on the contrary Neu4 inhibition increases neurite formation (*92, 93, 97, 98*). Total beta galactosidase activity was increased during the aging of the rat hippocampus in vitro and in vitro experiments (*102*). These results suggest

that a shift of ganglioside expression during development is regulated by changes of the expression level and activity of these key glycosyltransferases. Not only gangliosides but also other GSLs, such as GalCer and sulfatide, present drastic pattern shifts during development.
The multiple roles of GSL in regulating cellular functions, essential for the development and the homeostasis of the nervous system, can be explained by their ability to modulate the activity of plasma membrane via direct SL–protein or indirect (mediated by lipid rafts) lateral interactions (cis interactions), as discussed above (*2, 31, 83, 103-105*).

BIOSYNTHESIS

Glycolipid biosynthesis in animals requires the intracellular formation of the membrane anchor (*106*) and the subsequent addition of single carbohydrate residues (*27*). Both events are coupled to intracellular movement of metabolic intermediates and final products to the plasma membrane (*29*). The combinatorial variety of naturally occurring glycosphingolipids can be largely attributed the combination of glycosyltransferase activities found in different species and cell types.

CERAMIDE

The *de novo* biosynthetic pathway of sphingolipids starts at the cytosolic face of the endoplasmic reticulum (ER), where enzyme activities responsible for the reaction sequence leading to the formation of ceramide are localized. Ceramide synthesis (Figure 5) is due to the condensation of the amino acid L-serine with a fatty acyl coenzyme A, usually palmitoyl coenzyme A, to 3-ketosphinganine and it is catalysed by the enzyme serine palmitoyl-transferase (SPT) (*107-109*). In the following NADPH-dependent reaction, 3-ketosphinganine is reduced to D-*erythro*-sphinganine by 3-ketosphinganine reductase (*110*). Sphinganine is subsequently acylated to dihydroceramide by a *N*-acyltransferase (*111-113*). The major part of the dihydroceramide pool is desaturated to Cer in the dihydroceramide desaturase reaction (*114-116*).
Ceramide synthase is able to use as substrate both sphingosine and sphinganine with similar efficiency (*117*), so ceramide can be also formed by N-acylation of sphingosine produced by the catabolism of complex sphingolipids.

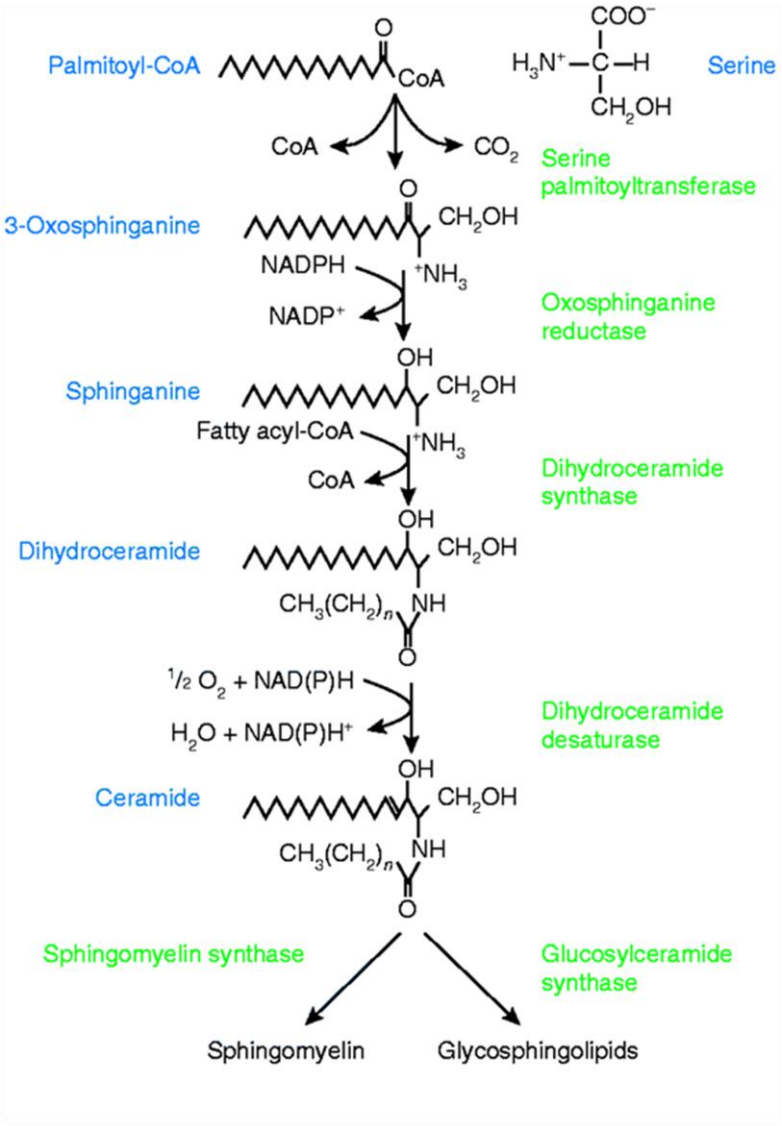


Fig. 5: De novo ceramide biosynthesis

Ceramide is also generated during the catabolism of complex sphingolipids (Figure 6). The catabolism occurs in the lysosomes by the action of glycohydrolases that remove single saccharidic units from the not reducing extremity of the oligosaccharides head group of GSL. Ceramide can be also produced by the SM hydrolysis is catalyzed by the action of sphingomyelinases (SMases) (*118, 119*). Different forms of SMases are known, which are characterized by specific optimum pH, subcellular localization, cation dependence.

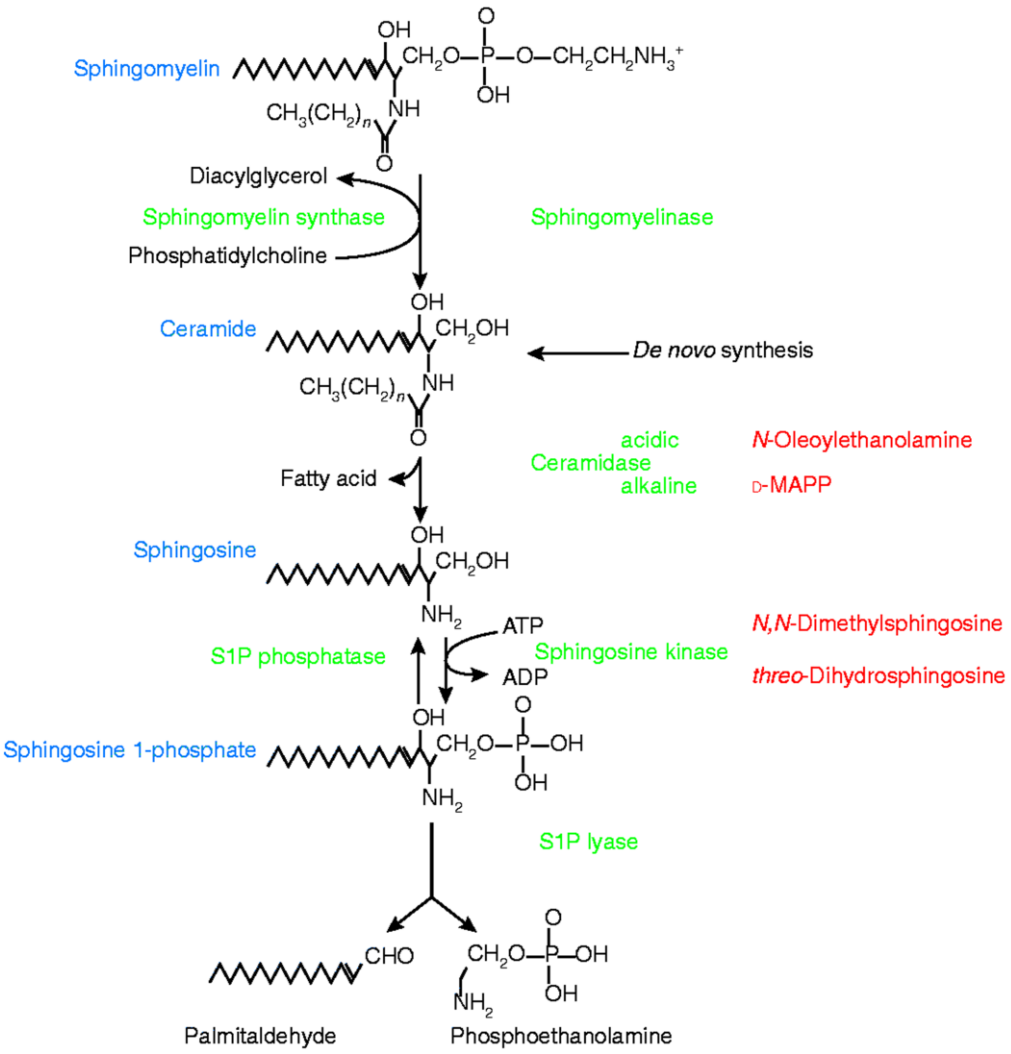


Fig. 6: ceramide from catabolism

SPHINGOMYELIN AND SPHINGOLIPIDS

The neo-synthesized ceramide could reach directly the membrane or it is used as common precursor for glycosphingolipid or sphingomyelin biosynthesis. In both cases the ceramide reaches the Golgi apparatus by a not well defined mechanism (*29*), capable to address the synthesis of SM or GSL. Synthesis of SM occurs via addition of phosphocholine group to the hydroxyl group in position 1 of the sphingoid base of the ceramide. In the case of the biosynthesis of glycosphingolipids, different membrane-bound glycosyltransferases are responsible for the sequential addition of sugar residues to the hydroxyl group in position 1 of the ceramide, leading to the growth of the oligosaccharide chain.

Glucosylceramide (GlcCer) is the first glycosylated product, formed by a ceramide glucosyltransferase activity localized at the cytosolic side of the early Golgi membrane (*120*).

Glucosylceramide can either directly reach the plasma membrane (*121*), presumably transported in a non-vesicular way (*121*), or be translocated to the luminal side of the Golgi, where it is further glycosylated by other glycosyltransferases located in this cellular district to generate more complex glycosphingolipids. Lactosylceramide(LacCer), the common precursor for the GSL series found in vertebrates, is formed by the addition of a galactose moiety from UDP-Gal to GlcCer catalysed by galactosyltransferase. The enzyme has been purified and cloned from rat brain (*122*). LacCer formation and also the reactions leading to higher glycosylated lipids occur on the luminal leaflet of Golgi membranes (*123*). Neo-synthesized glycosphingolipids move through the Golgi apparatus to the plasma membrane following the mainstream exocytotic vesicular traffic (Figure 9).

Hundreds of sphingolipid molecular species, differing in their polar head group, sphingoid base, and fatty acyl moiety, have been described. Referring to the polar head group it is possible to distinguish a very wide group, inside the large family of the sphingolipid, represented by glycosphingolipids (GSLs) that are amphipathic molecules composed of a hydrophilic carbohydrate chain and a hydrophobic ceramide moiety (*124*). GSLs containing one or more sialic acid residues in the carbohydrate chain are referred to as gangliosides. Based on the sequences of the core carbohydrate residues, GSLs are classified into a number of series (including gala-, ganglio-, isoganglio-, lacto-, neolacto-, lactoganglio-, globo-, isoglobo-, and muco-series) (Figure 7). Structural diversity in their carbohydrate chains is a hallmark of GSLs. At present, 172 neutral GSLs, 24 sulfated GSLs, and 188 gangliosides with variations in the carbohydrate chain have been reported in a variety of vertebrate tissues and organs (*125*). This complexity is increased many fold when heterogeneity in the lipophilic components is taken into consideration.

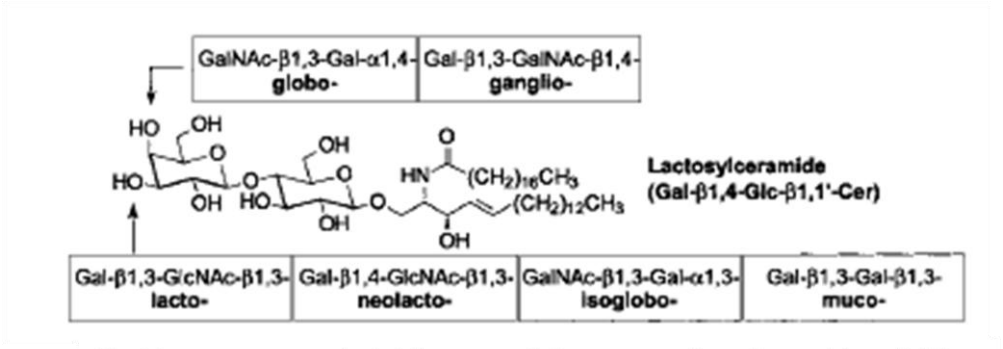


Fig. 7: structures and trivial names of the mammalian glycosphingolipid series derived from lactosyceramide

COMPLEX GANGLIOSIDES

A GSL series that is especially abundant on neuronal cells is the ganglio series. The biosynthesis of sialic acid-containing GSLs of this series, the gangliosides, is catalysed by glycosyltransferases in the lumen of the Golgi apparatus (*27, 126, 127*). Gangliosides are structurally and biosynthetically derived from LacCer. LacCer and the hematosides GM3, GD3 and GT3, serve as precursors for complex gangliosides of the o-, a-, b- and c-series (Figure 8). In adult human tissues, gangliosides from the o- and c-series are found only in trace amounts. The transferases that catalyse the first steps in ganglioside biosynthesis show high specificity towards their glycolipid substrates, i.e. for the formation of LacCer, GM3 and GD3. The relative amounts of these glycosphingolipids in the steady state seems to determine the amount of o-series glycosphingolipids, which are derived only from LacCer, a-series gangliosides which are only derived from ganglioside GM3, and b-series gangliosides which are only derived from ganglioside GD3. Sialyltransferases I and II are much more specific for their glycolipid substrates than sialyltransferases IV and V, or than galactosyltransferase-II and GalNAc transferase. It was assumed that different transferases catalyse the formation of homologous gangliosides of different series.

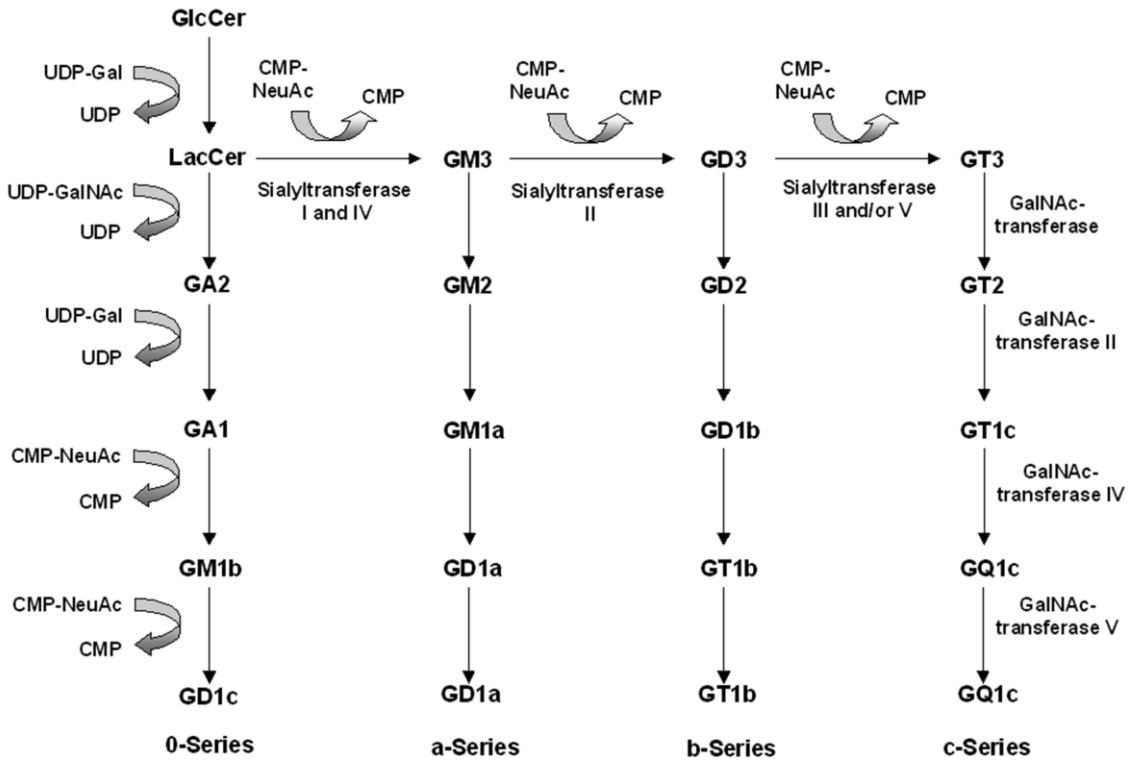


Fig. 8: scheme of ganglioside biosynthesis

Recent results suggest that in some cell types recycling of partially degraded GSLs in the recovery pathway is of considerable importance for the synthesis of GSLs (*123*). The regulation of GSL biosynthesis, as well as the factors that account for stable GSL patterns on individual cell surfaces and the characteristic changes observed during development and malignant transformation, is currently under active investigation (*128, 129*). A dominant regulatory role can be ascribed to glycosyltransferase activities, which appear to be controlled mainly at the transcriptional level. In addition, epigenetic regulation mechanisms are considered. In vitro, feed back control of several glycosyltransferases either by their reaction product or the final product within the series has been observed.

Furthermore, the enzymes activities were found to be dependent on their phosphorylation status and the pH value. On the other hand, the extent of cell surface sialylation depends on the activity of UDP-GlcNAc 2-epimerase, the key enzyme in sialic acid biosynthesis. In contrast to the great number of inherited human diseases attributed to disorders in GSL catabolism, nothing is described about defects in GSL biosynthesis. It may be assumed that incorrect GSL patterns on the cell surface resulting from disorders in GSL biosynthesis would severely impair morphogenesis and prevent embryonic development. Recently, the targeted

disruption of the glucosylceramide-synthase gene in mice led to impaired embryogenesis and demonstrated conclusively the critical necessity of GSLs for embryonic development and tissue differentiation (*130, 131*).

CATABOLISM

Another important point of regulation of plasma membrane sphingolipids composition is the lipidic degradation that occurs in the acidic compartments of the cells, the lysosomes, where sphingolipids are transported by the endocytic vesicular flow through the early and late endosomal compartment to be catabolyzed (Figure 9).

Lysosomal glycosidases sequentially cleave off the sugar residues from the non-reducing end of their glycolipid substrates. All the enzymatic steps of the degradative process require an acidic pH inside the organelle, a condition that is warranted by the action of a proton pump that brings H^+ into the lysosomes (*132*).The resulting monosaccharides, sialic acids, fatty acids and sphingoid bases can leave the lysosome and be used within recovery processes or can be further degraded.

Portions of the plasma membrane enriched in GSLs bud into coated pits that are internalized, uncoated and subsequently fuse with early endosomes.

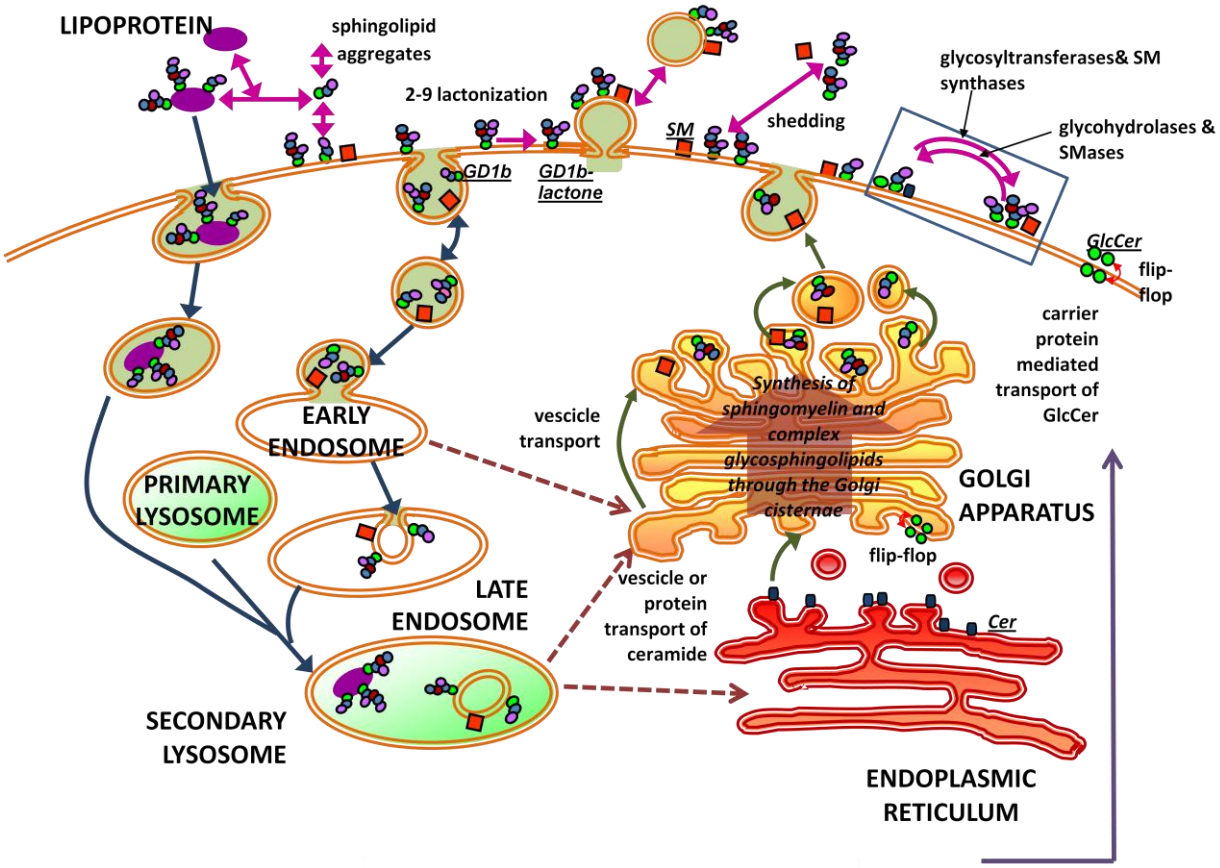


Fig. 9: intracellular sphingolipid metabolism

Degradation of sphingolipids derived from the cell surface that become part of the limiting membrane of endosomes and lysosomes is prevented by the thick glycocalyx, which protects the membrane from the attack of the lipid-degrading enzymes present in the lysosome (*133*). This glycocalyx is composed of the carbohydrate part of lysosomal integral membrane proteins and lysosomal associated membrane proteins (*134*) which are highly N-glycosylated with lactosamine units. The enzyme/activator system required for degradation cannot reach their substrates through this glycocalyx. This explains why glycosphingolipids of the perimeter membrane are much more resistant towards degradation than plasma membrane-derived GSL (*135*).

Experimental evidences have recently shown that the major part of GSL catabolism occurs on small intra-endosomal and intra-lysosomal compartment (*136*). Additional evidence for the proposed route came from experiments in human fibroblasts: biotin-labelled ganglioside GM1 derived from the plasma membrane is mainly targeted to intra-lysosomal structures and much less to the lysosomal perimeter membrane (*137, 138*). If degradation occurs on the surface of these intra-lysosomal membrane structures, lipid composition and lateral

pressure should influence the degradation process. Analysis of these molecular details supports the initial hypothesis of GSL degradation at intra-lysosomal membrane structures.

According to the model discussed above, the degrading lysosomal enzymes cleave substrates that are part of intralysosomal membrane structures. In the absence of detergents that solubilize the lipids, GSL with carbohydrate chains of one to four residues (*139*) are not sufficiently accessible to the hydrolytic water-soluble enzymes in the absence of a membrane perturbing activator protein. For this reason the intralysosomal degradation of most, if not all, glycosphingolipids requires, besides exoglycohydrolases, effector protein molecules named "sphingolipid activator proteins (SAPs, or saposines)" (*132*).

The sequence of sugar removal from gangliosides within the lysosomes is as follows: firstly, multi-sialogangliosides are transformed by the lysosomal sialidase into the corresponding mono-sialogangliosides GM1 and GM2 (which are resistant to this enzyme) (Figure 10). The linkage of sialic acid in sialic acid containing molecules is hydrolyzed by several different sialidases. Neu1 is the lysosomal enzyme, known from long time to be the enzyme for the catabolism of sialo-compounds. Neu2 is prevalently located in the cytosol whereas the two isoforms of Neu4, the short and the long, are associated to internal membranes and mitochondria, respectively. However the role of Neu2 and Neu4 is still not clear *in vivo*. Neu3 is the plasma membrane sialidase. Only from 2000 Neu3 structure was elucidated, resulting distinct from that of the other known sialidases, being cloned in human (*140*), bovine (*141*) and mouse (*93*).

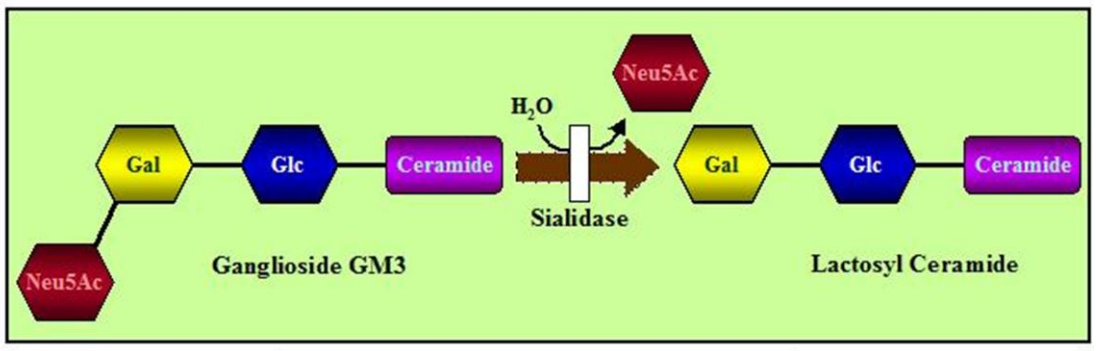


Fig. 10: reaction catalyzed by sialidase

From GM1, galactose is then removed by a β-galactosidase, working in the presence of either the GM2-AP or SAP-B (*139*), to produce GM2 (Figure 11). β-galactosidase are glycohydrolytic enzymes that removes β-galactosides from various sialo derivatives

catalyzing the hydrolyses of β-galactosides into monosaccharides. Substrates of different β-galactosidases include ganglioside GM1, LacCer, lactose, and various glycoproteins.

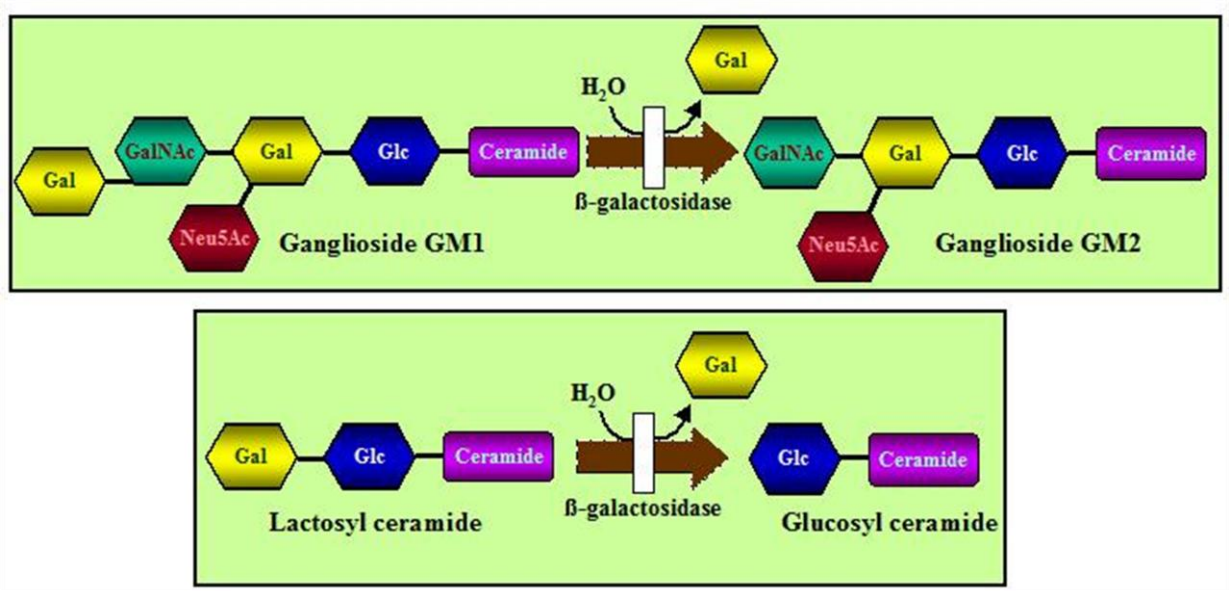


Fig. 11: reaction catalyzed by β-galactosidase

The resulting ganglioside GM2 is cleaved to ganglioside GM3 and *N*-acetyl-galactosamine only by the β-hexosaminidase isoenzymes Hex A which requires the GM2-AP, an activator that is essential for the *in vivo* degradation of the GM2 gangliosides (Figure 12).

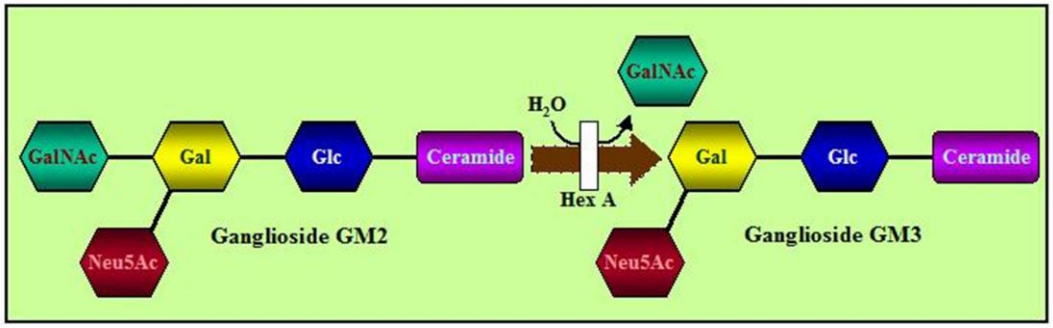


Fig. 12: reaction catalyzed by hexosaminidase

The heterodimer Hex A (αβ) has two active sites that differ in their substrate specificity (*142-144*) and its presence in the external leaflet of PM has been demonstrated in cultured fibroblasts (*145*). The other subunit combinations in the hexosaminidase enzymes are Hex B (ββ-homodimer) and Hex S (αα-homodimer). The catalytic centre of the β-subunit cleaves

predominantly neutral sugars chains with terminal *N*-acetyl-β-D-galactosaminyl and *N*-acetyl-β-D-glucosaminyl residues. Towards uncharged substrates, the active site on the α-subunit is less active than the β-unit, but cleaves sugars from negatively charged substrates (*146*). The enzyme binds another molecule called GM2 activator protein (Figure 13) in order to form this complex that can hydrolyzes GM2 ganglioside removing β-1,4-linked N-acetylhexosamine residue.

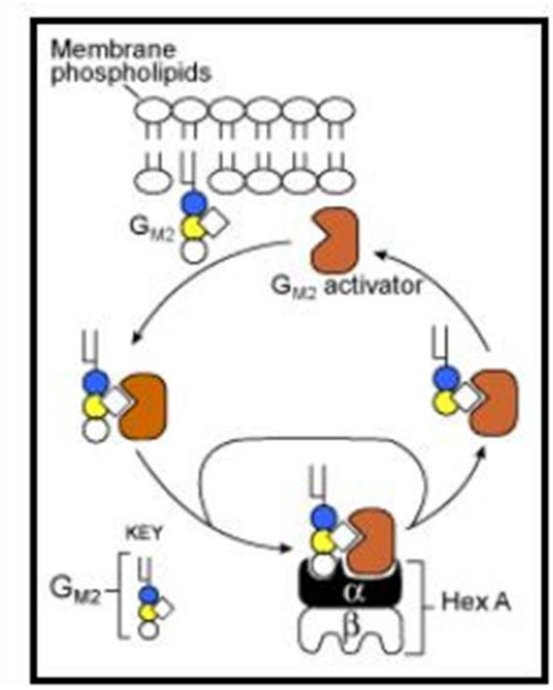


Fig. 13: GM2 ganglioside degradation by β-hexosaminidase A

In some cells and animals, sialic acid is removed from GM1 and GM2 by a specific sialidase (GM1- and GM2- sialidase) producing the corresponding a-sialo derivatives GA1 and GA2, that, by the action of β-galactosidase and β-hexosaminidase or only β-hexosaminidase respectively, are converted to LacCer. The presence of GM2- and GM1-sialidase, first described in 1971 (*147*), was definitely confirmed later (*148-150*). LacCer is produced also directly from GM3 by the action of a sialidase which cleaves this ganglioside into LacCer and sialic acid in a reaction stimulated by SAP-B (*151*). LacCer is then degraded to ceramide by the sequential action of a β-galactosidase (in the presence of either SAP-B or –C) and β-glucosidase (*152*) (which is a glucosidase enzyme which acts upon β-1,4 bonds linking two glucose) (Figure 14).

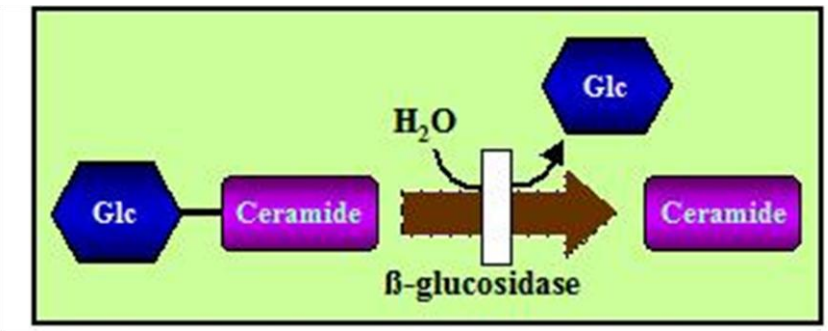


Fig. 14: reaction catalyzed by β-glucosidase

Ceramide is cleaved by acid ceramidase in the presence of SAP-D (*153*) into sphingosine and a fatty acid. Together with the other cleavage products, these two metabolites are able to leave the lysosome. An alternative pathway for ganglioside/glycosphingolipid degradation is the splitting of the β-glucosidic linkage between glucose and ceramide, with the formation of ceramide and the oligosaccharide. The enzymes catalysing this reaction, named "endoglycoceramidases" or "ceramide glycanases" (*154-159*) appear to require, or to be markedly activated, by specific activator proteins whose action would be essential under in vivo conditions (*154*). Endoglycoceramidases have been found to occur in some bacteria (*154*) and leeches (*155*). Although described to occur in lactating mammary glands of rodents (*157*), the presence of this enzyme in vertebrate and, particularly, mammalian tissues is yet to be definitely assessed.

For the non-glycosylated sphingolipids, like ceramide and SM, non-lysosomal degradation steps are known which apparently do not need the assistance of an activator protein. SM is cleaved to Ceramide and phosphorylcholine. Ceramide later on is degraded into sphingosine and a fatty acid by ceramidases of different subcellular localization. In the cytosol, sphingosine can be phosphorylated to sphingosine-1-phosphate or can be re-acylated to Ceramide. These highly regulated degradation processes occur at several subcellular places to produce various signal molecules (*132, 160*). Enzymes were also described that are capable to remove the fatty acid moiety from several sphingolipids (sphingomyelin, gangliosides and some neutral glycosphingolipids) producing the corresponding lyso-derivatives (*160, 161*). These enzymes, known as sphingolipid ceramide N-deacylases, were detected in bacteria. No evidence was yet provided for their occurrence in vertebrates. However, it is known that in the brain of patients suffering from some sphingolipidoses

where there is accumulation of lyso-glycosphingolipids (*162*) these accumulated compounds seems to be the products of proteins having this kind of enzymatic activity. These products can leave the organelle and re-enter the biosynthetic pathway or serve as energy sources and be further degraded.

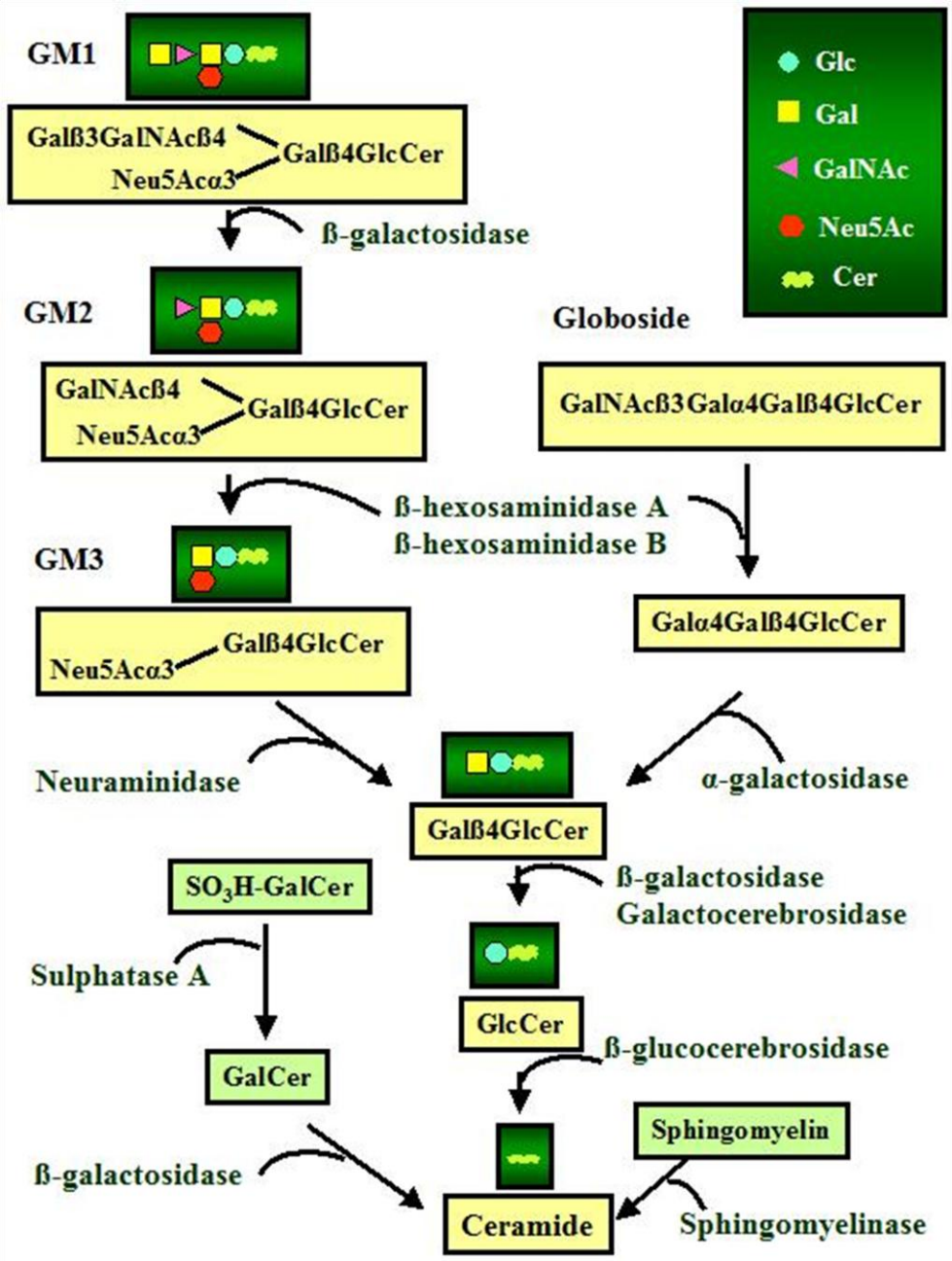


Fig. 15: catabolic pathway of GSLs

GLYCOHYDROLASES

Glycohydrolases are a widespread group of enzymes involved in GSL metabolism, that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety also called as glycosidases. The glycohydrolases are ubiquitous enzymes, being found in lysosomes, plasma membranes, cytosol and blood plasma (*163-167*). They are involved in the biosynthesis and degradation of glycogen in the body. Together with glycosyl transferases, glycosidases form the major catalytic machinery for the synthesis and breakage of glycosidic bonds.

Glycohydrolases are found in essentially all domains of life. In bacteria and prokaryotes, they are found both as intracellular and extracellular enzymes largely involved in nutrient acquisition. One of the important occurrences of glycohydrolases in bacteria is the enzyme β-galactosidase (LacZ), which is involved in regulation of expression of the Lac-operon in E. Coli. In higher organisms glycohydrolases are found within the endoplasmic reticulum and Golgi apparatus where they are involved in processing of N-linked glycoproteins, and in the lysosomes as enzymes involved in the degradation of carbohydrate structures. Deficiency in specific lysosomal glycohydrolases can lead to a range of lysosomal storage disorders that result in developmental problems or death. The glycohydrolases are involved in the biosynthesis and degradation of glycogen in the body.

In each subcellular location glycohydrolases may be present in different forms, as shown by N-acetyl-L-D-glucosaminidase and sialidase (*168, 169*). The insertion of glycohydrolases into the plasma membrane is assumed to occur through a transmembrane hydrophobic domain. However, examples are known of other types of anchoring (*170*).

Glycohydrolases are enzymes that catalyze the hydrolysis of O- or S-glycosides, they can also be classified according to the stereo chemical outcome of the hydrolysis reaction: thus they can be either retaining or inverting enzymes (*171*). Glycohydrolases can also be classified as exo- or endo- acting, dependent upon whether they act at the (usually non-reducing) end or in the middle, respectively, of an oligo/polysaccharide chain.

Glycohydrolases may finally also be classified by sequence based methods. Sequence-based classifications are among the most powerful predictive method for suggesting function for newly sequenced enzymes for which function has not been biochemically demonstrated. A classification system for glycohydrolases, based on sequence similarity, has led to the definition of 85 different families (*145, 172, 173*). This classification is available on the CAZy

(Carbohydrate-Active EnZYmes) web site. The database provides a series of regularly updated sequence based classification that allow reliable prediction of mechanism (retaining/inverting), active site residues and possible substrates. Based on three dimensional structural similarities, the sequence-based families have been classified into 'clans' of related structure. Recent progresses in glycosidase sequence analysis and 3D structure comparison have allowed the proposal of an extended hierarchical classification of the glycohydrolases.

THE REGULATION OF GSL COMPOSITION AT THE PLASMA MEMBRANE

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 vescicular transport of neo-biosynthesized sphingolipids from the endoplasmic reticulum and the Golgi apparatus to the plasma membranes (reviewed in (*174*)). Changes in the activities of enzymes of the biosynthetic pathway have been associated with the changes in GSL expression that are correlated 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~~ on restricted plasma membrane areas (Figure 16):

1) Both catabolic and biosynthetic enzymes for sphingolipids have been found associated with the plasma membranes. “Signaling” sphingomyelinases are resident in or translocated to the plasma membrane, being able to convert plasma membrane sphingomyelin into ceramide (*175, 176*). Conversely, a plasma membrane-associated sphingomyelin synthase enzyme activity (SMS2), genetically distinct from the Golgi enzyme, has been identified (*177*). 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 (*93, 140, 141*), 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 (*1, 178, 179*). Moreover,

the presence of sialyltransferase activities at the cell surface has been also reported (*180-184*). 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 glycohydrolases such as β-glucosidase, β-galactosidase and β-hexosaminidase (*1, 185*) 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 (*186-188*), whose controlled release from specific glycolipid-enriched membrane areas could represent a further way to modify the lipid membrane domain composition and organization.

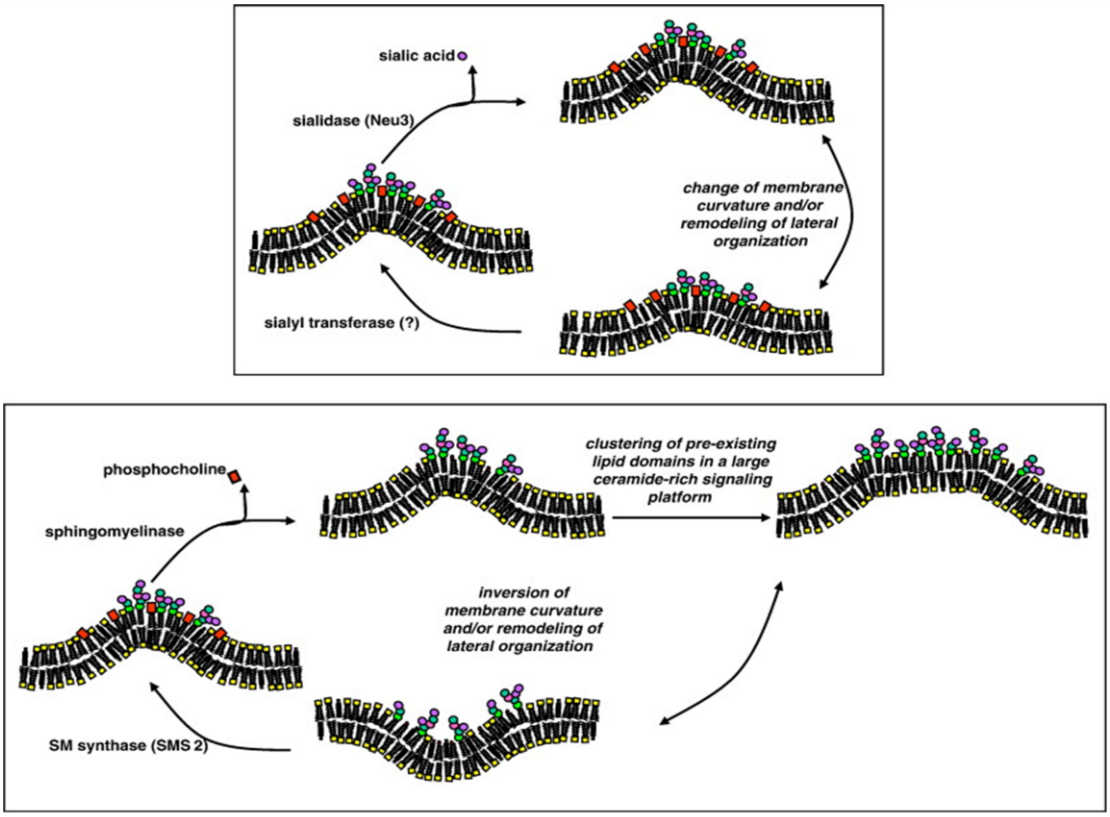


Fig. 16: Regulation of sphingolipid-enriched membrane domain composition by phosphorylation/dephosphorylation ("sphingomyelin cycle") and glycosylation/deglycosylation.

MEMBRANE GLYCOHYDROLASES

Plasma membrane glycoconjugates show changes during development and neoplastic transformation (*189, 190*) suggesting a specific role in cell–cell recognition. Glycosyl epitope changes include many different residues, such as GlcNAc, GalNAc in N-linked structures or sialyl residues in either protein N-linked, O-linked or lipid linked structures, so that cell surface glycohydrolases could be one of the natural candidate for plasma membrane glycosylation modification.

For this reason, although in the past little attention was paid to glycohydrolases acting on cellular compartment different from lysosomes, there is now increasing interest on plasma membrane associated glycohydrolases because on this compartment they should be involved, together with glycosyltransferases, in GSL oligosaccharide modification processes regulating cell to cell and/or cell–environment interactions in both physiological and pathological mechanisms (*179, 191, 192*) .

Glycohydrolases are the enzymes involved in GSL metabolism. The presence of glycohydrolases on the plasma membrane (PM), where they can be involved in the modifications of the cell surface GSLs or of glycoproteins, has been demonstred (*3*). This class of enzymes are taken into account as they can participate in the modulation of signal transduction process.

Some of the plasma membrane associated glycohydrolases are identified and their activity has been studied for characterisation. They are

- ✓ Hexosaminidase
- ✓ Sialidase or Neuraminidase
- ✓ β-galactosidase
- ✓ β-glucosidase
- ✓ Sphingomyelinase

The presence of β-hexosaminidase A in the external leaflet of PM has been demonstrated in cultured fibroblasts (*185*). Immunological and biochemical characterization of the membrane-associated β-hexosaminidase indicated that this enzyme has the same structure of that in lysosomes.

The linkage of sialic acid in sialic acid containing molecules is hydrolyzed by several different sialidases. Taking in account all the available data, Neu3 can be considered an ubiquitous enzyme being expressed at different levels in all the plasma membrane of tested normal and pathological human tissues such as human brain (*95*) normal and colon rectal carcinoma tissue, hepatic tumor and kidney carcinoma (*193-197*). In addition its expression and activity were also found in normal and pathological cell lines such as erytroid and erytroleukemic cells (*198-200*), human fibroblasts (*201*), rat and mouse neurons, neuroblastoma cells (*202*), human breast ductal cancer T47D cells, colon carcinoma CaCo2 cells, human colorectal adenocarcinoma HT29 cells, different type of ovarian cancer cells, human cervix adenocarcinoma HeLa cells. (*197*)

An increase of Neu3 activity modifies the cell surface ganglioside composition by shifting Gg4 and Gg3 polysialylated species to monosialoderivatives, and GM3 to lactosylceramide, with deep consequences on neuronal differentiation and apoptosis in normal and pathological cells (*1, 197, 203*). In cultured hippocampal neurons, the activity of Neu3 locally regulated GM1 surface levels, and was essential for axonal growth and regeneration after axotomy (*98*). Neu3 and gangliosides co-localize in lipid rafts (*178*). The non-random 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 glycosphingolipid-based signalling units. Remarkably, Neu3 is capable to modulate the cell surface glycolipid composition because of *trans* interactions, thus modifying the surface of neighboring cells (*179*).

Plasma membrane associated β-galactosidase has been found in all cell lines we studied for the activity of Neu3. In human fibroblasts its expression is up regulated by Neu3 (*1*). The β-galactosidase activity was measured during neuronal cell differentiation and aging, in the total cell lysate and in the plasma membranes from rat cerebellar granule cells. Both these activities resulted up regulated during cell differentiation. As expected the β-galactosidase activity associated to the cell plasma membranes was much less than that found in total cell homogenate. The total cell activity remained constant during differentiation, then increased 4 fold along aging in culture. Instead, cell surface activity increased 10 fold during differentiation, to then duplicate during the neuronal senescence (*204*). β-galactosidase activity has been proposed as senescent marker of different cell lines (*205-208*). The information on the behavior of plasma membrane associated enzyme in rat cerebellar

granule cells suggests that its activity could be used as a good marker for both neuronal differentiation and neuronal aging. 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 has been verified the presence of a β-galactosidase activity which displays a *trans* activity, with no necessity of detergents or activator proteins, on lactosyl ceramide immobilized on cell culture plates, suggesting that on the cell surface is present at least an enzyme having a β -galactocerebrosidase-like activity (*3*).

Concerning β-glucosidase, at least three different enzymes have been described: a β-glucocerebrosidase (GBA1) sensitive to the inhibition of Conduritol B Epoxide (CBE) normally described associated to the lysosomes (*209*), a cytosolic β-glucosidase (*210*) and a non-lysosomal β-glucosylceramidase (GBA2). GBA2 has been found associated to endosome vescicles or to the plasma membrane. It is insensitive to CBE whereas is specifically inhibited by N-(5-adamantane-1-yl-methoxy)pentyl)-deoxynojirimycin (AMP-DNM) (*211, 212*). Recently it has been described that a CBE-sensitive β-glucosidase activity is present in the plasma membranes (*3*). Both CBE-sensitive and CBE-insensitive β-glucosidase activities were found associated to the cell surface of rat cerebellar granule cells in culture, where they progressively increase along the differentiation and the aging process (*204*).

Total β-glucosidase activity associated to the plasma membranes of human living fibroblasts works on natural substrates *in trans* fashion (*3*) and the activity is connected to Neu3 activity. In fact, in human fibroblasts overexpressing Neu3, the β-glucosidase activity associated to the plasma membrane was much higher of that found in normal fibroblasts. Surprisingly this is mainly due to an up-regulation of the CBE-sensitive β-glucosidase (*3*).

Three different SMases are available in eukaryotic cells (*213*): secreted SMase, acid SMase and neutral SMase. Secreted SMase exerts its activity in the extracellular environment. Acid SMase are principally located in the lysosomes and few evidences reports about its association to PM. Neutral sphingomyelinase identify a family of different enzymes that catalyze the hydrolysis of SM into ceramide at pH 7.4 and are Mg^{2+}-dependent enzymes. Among the three recently cloned mammalian N-SMases, only nSMase2 and nSMase3 display *in vivo* activity. nSMase3 is reported to localize to the endoplasmic reticulum and the Golgi compartment (*214, 215*) while the subcellular localization of nSMase2 remains not fully determined. nSMase2 was first described as a Golgi-associated protein potentially involved in the Golgi secretory vesicle trafficking (*216*), however other studies have indicated a PM

localization of overexpressed GFP-, FLAG- or V5-tagged-nSMase2 in various cancer cell lines (*217-219*). This enzyme is characterized by the presence of two palmitoylation sites that were reported to be important for PM localization (*220*). Furthermore, stimuli such as TNFα, H_20_2 and cell confluence seemed to induce nSMase2 translocation from the Golgi to PM lipid rafts (*213*).

Therefore the glycohydrolitic enzymes, responsible for the catabolism of the sphingolipids, are not only recovered in the classical cellular compartment deputed to the catabolism (the lysosomes) but are also associated to other cellular district. In some case the same lysosomal enzyme has been found associated to other compartments, as is the case of the β-hexosaminidase A associated to the plasma membrane; in some others, enzymes codified by different genes have been found in different regions of the cell. This last is the case of the sialidases Neu2, Neu3 and Neu4 with respect to the lysosomal enzyme Neu1, or the case of the non-lysosomal β-glucosylceramidase GBA2.

Considering the presence of lysosomal glycohydrolases at the cell surface two aspects need to be clarified: how lysosomal deriving enzymes can reach the plasma membrane and how they can be active at the cell surface where the pH conditions are far from those of the lysosomes? It is known that a regulated fusion of lysosomes with the plasma membrane might represent a general mechanism of repairing for the plasma membrane (*221*), and could be responsible of the presence of lysosomal enzymes facing at the cell surface. However this enzymatic "translocation" could not be accidental and the fusion of lysosomal membranes with the plasma membrane could be the way for all the lysosomal glycolipid-metabolizing enzymes to reach the cell surface where, together with specific and different membrane associated enzymes, play an active role in remodeling the glycolipid content and pattern of the external leaflet of the plasma membrane.

At the cell surface the pH conditions are usually considered near to the neutrality, whereas the lysosomal pH is much more acidic. For some glycohydrolases described associated to the plasma membrane has been evaluated the optimum of pH to which they play their catalytic activity. Both the β-hexosaminidase A and Neu3 (respectively codified by the same and by a different gene with respect to the corresponding lysosomal enzyme) has been found working in acidic condition (pH 4.5 and pH 4.2 respectively). This information suggests that modulation systems, capable to modify the pH conditions, even locally, could be present at the cell surface. One hypothesis for this theory may be the action of proton pumps present

at the plasma membrane level for which has already been demonstrated a role in the acidification of the tumor extracellular environment (*222-224*).

TRANSPORTERS/EXCHANGERS PRESENT AT PM LEVEL

The induction and maintenance of intracellular alkalization and its subsequent extracellular, interstitial acidosis on intratumoral dynamics have been repeatedly implicated as playing an essential, direct and pivotal role both in cell transformation as well as in the active progression and maintenance of the neoplastic process. Both *in vitro* cell-culture studies and *in situ* tumor spectroscopic studies utilizing the ^{31}P isotope have reported that tumor cells have alkaline intracellular pH (pHi) values (7.2–7.7 compared with 6.99–7.20 in normal tissues) and acidic interstitial extracellular pH (pHe) values (6.2–6.8 compared with 7.3–7.4) (*222*). So, the tumor extracellular environment is more acidic than the intracellular environment, creating a reversed pH gradient across the cell membrane that increases as the tumor progresses and it is considered to be one of the main characteristics defining the molecular energetic of tumors, regardless of their pathology and genetic origins (*225*). There are now ample data demonstrating that the aberrant regulation of hydrogen ion dynamics (the lower the oxygen level, the more protons H^+ accumulate) leading to this reversed proton gradient is driven by a series of proton export mechanism from blood vessels to the necrotic area that underlie the initiation and progression of the neoplastic process.

Continual re-adjustment of the pHi is essential since a variation of 0.1 units can disrupt multiple biological functions including ATP production, protein synthesis, cell proliferation, migration and apoptosis through caspase activation. A variety of constitutively expressed membrane-associated systems are involved in the regulation of the pHi of all mammalian cells (Figure 17):

- ✓ the family of growth factor-activated **NHEs** (9 isoforms), that are Na^+/H^+ exchangers,
- ✓ the vacuolar-type H^+-ATPase (**V-ATPase**), that requires ATP to actively extrude two H^+,
- ✓ the **MCTs** (MCT1, MCT2 and MCT4), that transport lactic acid bidirectionally,
- ✓ the NA^+-$HCO3^-$ co-transporters (**NBCs**) and the Cl^--$HCO3^-$ exchangers (**AEs**) that, in the opposite direction to H^+ extrusion, favour $HCO3^-$ influx and thus contribute to cytoplasmatic alkalinisation,

✓ the family of carbonic anhydrases (**CAs**) and particularly, the cytosolic CAII

have been shown to play a key role in the regulation of the physiological pH by catalysing the hydration of carbon dioxide to protons and bicarbonate ions (*226*).

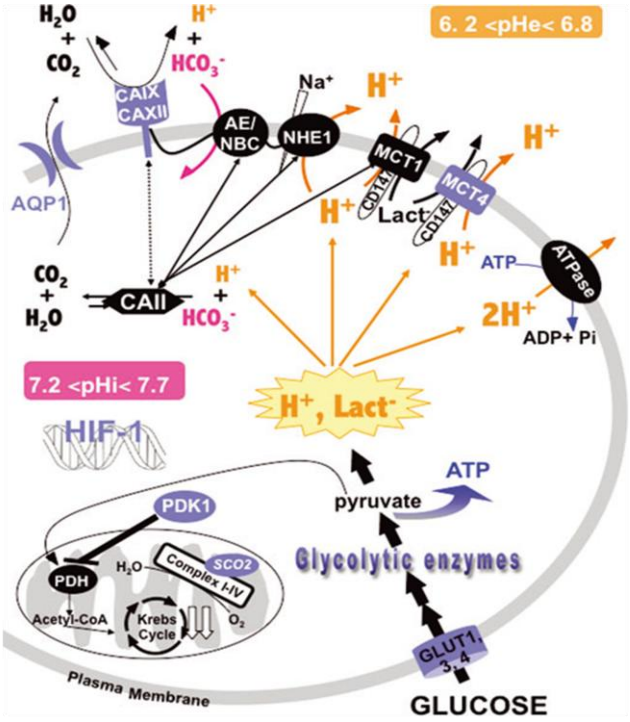


Fig. 17: Up-regulation of glycolysis and pHi regulation: hallmarks of invasive cancers. Image from *"Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer", Chiche et al., J. Cell. Med. Vol 14, No 4, 2010 pp. 771-794.*

THE Na^+/H^+ EXCHANGER NHE1

The Na^+/H^+ exchanger NHE1 is a member of a family of secondary active acid extruders that mediates the 1:1 exchange of extracelluar sodium for intracellular protons across the cell membrane. Through NHE1 action, the inwardly directed sodium gradient can drive the uphill extrusion of protons, alkalinize intracellular pH (pHi) and acidify the extracellular pH (pHe). NHE1 is expressed ubiquitously in non-epithelial cells and on the basolateral membrane of epithelial cells. It is composed of 12 transmembrane segments and a long carboxy-terminal cytoplasmic tail that has a role in both its regulation and function (Figure 24). The first functions of NHE1 to be identified were its role in regulating pHi homeostasis, cell volume and proliferation in response to growth factor stimulation. One of the important features of NHE1 is its exquisite sensitivity to pHi. When pHi drops below a threshold level NHE1 is

activated through an internal allosteric proton-binding regulatory site. This pHi sensitivity determines the activity set point of NHE1 (that is, the pHi at which NHE1 is quiescent) and, in normal cells, this set point is at the physiological, resting pHi. Neoplastic transformation increases the affinity of the proton-regulatory site, thereby increasing the sensitivity of NHE1 to the normal pHi, constitutively activating it and raising pHi. NHE1 in tumor cells is always active and these cells can have pHi values as high as 7.7. Through binding to the actin-binding protein VIL2 (villin 2, a member of the ezrin, radixin and moesin (ERM) family), NHE1 can directly regulate cytoskeletal dynamics independently of its ion-transporting capabilities, which permits the separation of the downstream functions of NHE1 into two groups. The first consists of those regulated by its ion translocation (Figure 18a), the second consists of those regulated by its ability to directly bind the ERM family and regulate cortical cytoskeleton dynamics by anchoring the cortical cytoskeleton to the plasma membrane (Figure 18b). Recently, NHE1 was also proposed to be able to act as a scaffolding protein through its direct association with a number of signaling proteins. These three different modes of regulation make NHE1 an important membrane-bound integrator for many signaling networks and cellular processes (*222*).

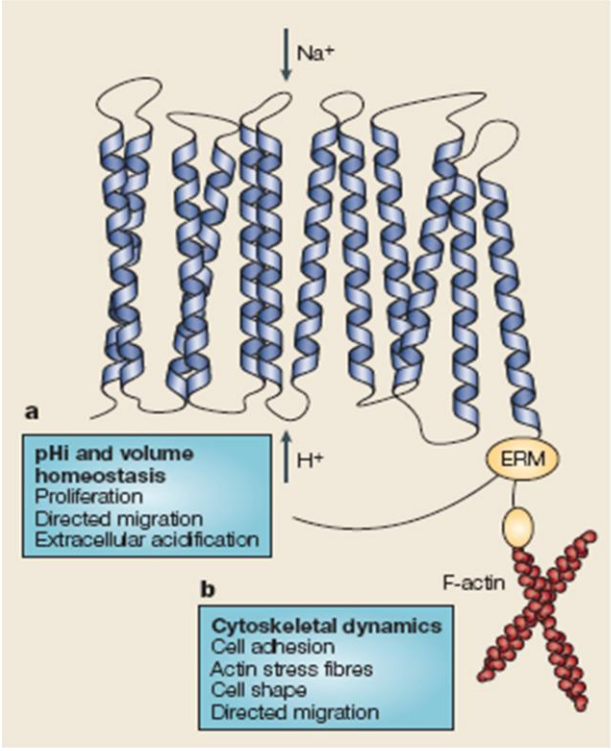


Fig. 18: The Na+/H+ exchanger NHE1.
Image from *"The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis"* , Cardone et al., Nature Reviews Cancer Vol 5, 2005 pp. 786-795.

MATERIALS & METHODS

CHEMICALS AND MATERIALS

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus. Trypsin, KCl, reagents for cell culture, Conduritol B Epoxide (CBE), 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) and 4-Methylumbelliferyl-β-N-acetylglucosaminide (MUG) were from Sigma-Aldrich (St Louis, MO, USA). Basal Modified Eagle's, Minimum Essential Medium with Earle's Salt (MEM) Dulbecco Modified Eagle's (DMEM) medium and fetal calf serum were purchased from EuroClone (Leeds, UK). The following primary antibodies have been used: rabbit polyclonal anti-MAP2 (#4542), mouse monoclonal anti-TAU (#4019), rabbit polyclonal anti-PSD95 (#2507), rabbit polyclonal anti-Synapsin (#2312) from Cell Signaling Technology, Inc. (Danvers, MA, USA); mouse monoclonal anti-Neuroglycan C (N73720), mouse monoclonal anti-L1 (L30220) from BD Biosciences (San Jose, CA, USA), mouse monoclonal anti-β-actin (A5441) and mouse monoclonal anti-β-tubulin (T4026) from Sigma-Aldrich (St Louis, MO, USA). Rabbit polyclonal antibody to TAG-1 was kindly provided by Dr. Karagogeos (*57*). As secondary antibodies we used mouse (#7076) and rabbit (#7074) horseradish peroxidase-conjugate antibodies from Cell Signaling Technology, Inc. (Danvers, MA, USA).

[1-^{3}H]Sphingosine was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro[^{3}H]hydride (radiochemical purity over 98%; specific radioactivity 2 Ci/mmol).

GM1 was prepared by sialidase digestion of a bovine brain ganglioside mixture and purified (Acquotti *et al.* 1994). LacCer was prepared by acidic hydrolysis of GM1 followed by purification (Sonnino *et al.* 1978). GM3 was prepared from GM1 (*227*). [3-^{3}H(*sphingosine*)]LacCer (radiochemical purity over 99%, specific radioactivity 1.2 Ci/mmole), [3-^{3}H(*sphingosine*)]GM3 (radiochemical purity 98%, specific radioactivity 1.2 Ci/mmole), and [3-^{3}H(*sphingosine*)]GM1 (specific radioactivity, 2.3 Ci/mmole) used as substrates of enzymatic assays and as chromatographic standards were prepared as described (*228*). Highperformance silica gel thin-layer plates (HPTLC Kieselgel 60, 10 x10 cm) were purchased from Merck GmbH (Milan, Italy). AM-calcein was from Invitrogen (Carlsbad, CA, USA). Triton X-100 were from Merck (Darmstadt, Germany). 4-Methylumbelliferyl- β-D-galactopyranoside (MUB-Gal), 4-Methylumbelliferyl- β-Dglucopyranoside (MUB-Glc) and 4-Methylumbelliferone (MUB) were from Glycosynth (Warrington, UK). 4- Methylumbelliferyl-

β-N-acetylglucosaminide-6-sulphate (MUGS) was from Toronto Research Chemicals Inc. (North York, Ontario, CDN). 6-Hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (HMU-PC) was from Moscerdam Substrates (Oegstgeest, Netherlands).

The *N*-(5-adamantane-1-yl-methoxy)pentyl-deoxynojirimycin (AMP-DNM), specific inhibitor for the non-lysosomal β-glucosylceramidase (GBA2) was kindly provided by Dr. Aerts, University of Amsterdam (*212*).

CELL CULTURES

FIBROBLASTS

Normal human skin fibroblasts were obtained by the punch technique from normal children (age 6-12 month), cultured and propagated as described (*229*) in 75-cm^2 flask the enzymatic assays 96-wells microplate at a density of 24000 cells/well for the surface enzymatic assay, using Minimum Essential Medium with Earle's Salt (EMEM) supplemented with 10% FBS, 1% Glutamine and 1% Pencilin/Streptomycin.

NEURONAL GRANULE CELLS

Granule cells, obtained from the cerebellum of 8-day-old Sprague-Dawley rats, were prepared and cultured as described (*230*). Cells were plated in 60-mm dishes at a density of 3 x 10^6 cells/dish for the enzymatic assays on total homogenate or plated in 24-wells microplate at a density of 375000 cells/well for the surface enzymatic assay. In both cases cells were cultured with Basal Modified Eagle's Medium (BME) containing 10% heat-inactivated foetal calf serum. The animals were sacrificed by decapitation.

PROTEIN ANALYSIS

The amount of protein present in the cell lysates was determined by DC protein assay in according to the manufactures instructions.

ENZYMATIC ACTIVITIES AT THE CELL SURFACE

PM ASSOCIATED SMase

PM-associated SMase activity was determined in living fibroblasts plated in 96-wells microplate. The artificial substrate HMU-PC was solubilized in the medium at the final concentrations of 0.50 mM. Cell culture medium was removed and cells were washed with DMEM without phenol red. The artificial substrate solubilized in the opportune medium was then added to the cell monolayers and the incubation was performed at 37°C under very gently stirring. At different times (from 30 minutes to 1 hours) aliquots of the medium were fluorometrically analyzed adding 15 volumes of 0.25 M glycine, pH 10.7 and 0,2% Triton X100. 50 pmoles of Free Hexadecanoil-amino-MUB were used as standard.

PM ASSOCIATED β-Gal AND β-Glc

PM-associated β-galactosidase and β-glucosidase activities were determined in living cells plated in 96-wells microplate for fibroblasts and 24-wells microplate for neuronal granule cells. The artificial substrates MUB-Gal and MUB-Glc were solubilized in DMEM without phenol red at the final concentrations of 0.25 mM and 6 mM respectively. The pH of medium was differently adjusted at pH 5.1 for the β-galactosidase assay, and at pH 6.3 and pH 5.7 for the activity assays of the two different β-glucosidases, the CBE-sensitive β-glucosidase and the CBE insensitive β-glucocerebrosidase GBA2 respectively. Cell culture medium was removed and cells were washed with DMEM without phenol red. For the CBE-sensitive β-glucosidase and GBA2 assays cells were pre-incubated for 30 minutes at room temperature with 5 nM AMP-DNM in DMEM, pH 6.3 and with 1 μM CBE in DMEM, pH 5.7 respectively. The artificial substrates solubilized in the opportune medium were then added to the cell monolayers and the incubation was performed at 37°C under very gently stirring. At different times (from 30 minutes to 6 hours) aliquots of the medium were fluorometrically analyzed adding 15 volumes of 0.25 M glycine, pH 10.7. 100 pmoles of free MUB were used as standard.

PM ASSOCIATED β-Hex

PM-associated β-hexosaminidase activity was determined in living fibroblasts plated in 96-wells microplate. The artificial substrates MUG and MUGS were solubilized in DMEM without phenol red at the final concentrations of 3 mM. MUG is hydrolysed by both α- and β-subunits, while MUGS is hydrolysed exclusively by α-subunit. Cell-conditioned medium was removed and cells were washed with DMEM. The artificial substrate solubilized in the opportune medium was then added to the cell monolayers and the incubation was performed at 37°C under very gently stirring. At different times (from 30 minutes to 90 minutes) aliquots of the medium were fluorometrically analyzed adding 15 volumes of 0.25 M glycine, pH 10.7. 100 pmoles of free MUB were used as standard.

PM ASSOCIATED ENZYMES ACTIVITY AT DIFFERENT pH CONDITIONS

PM associated activity for each enzyme was also determined at different pH conditions.
The pH of DMEM without phenol red was adjusted respectively with HCl (in order to obtain media at pH 5.10, 5.45 5.70 and 6.20) and NaOH (in order to obtain media at pH 7.90 and 8.40). Each assay was then performed as described above.

ENZYMATIC ACTIVITIES ASSOCIATED TO THE MEDIUM

MEDIUM ASSOCIATED SMase ACTIVITY

SMase activity associated with the medium was determined on the fluorogenic substrate HMU-PC. Cells were incubated for 2 days in DMEM without phenol red. Aliquots of medium were transferred in a 96-well microplate to perform the enzymatic assays in triplicate. HMU-PC was solubilized at a final concentration of 0.50 mM in McIlvaine Buffer (0.1 M Citrate/0.2 M Phosphate) pH 4.0. The reaction mixtures were incubated at 37°C under gently shaking for 3 hours. The developed fluorescence was detected at different times transferring 20μl of the reaction mixtures in an opportune microplate and adding 280 μl of 0.25 M glycine, pH 10.7.

The fluorescence was detected by a Victor microplate reader (Perkin Elmer). Any released enzymatic activity was not measured.

MEDIUM ASSOCIATED β-Gal AND β-Glc ACTIVITY

β-galactosidase and β-glucosidase activities associated with the medium were determined on the fluorogenic substrates MUB-Gal and MUB-Glc. Cells were incubated for 2 days in DMEM without phenol red. Aliquots of medium were transferred in a 96-well microplate to perform the enzymatic assays in triplicate. MUB-Gal and MUB-Glc were solubilized at the final concentrations of 0.25 mM in McIlvaine Buffer (0.1 M Citrate/0.2 M Phosphate) pH 4.0, whereas MUB-Glc was prepared at the final concentration of 6 mM in the same buffer at pH 5.2. The reaction mixtures were incubated at 37°C under gently shaking for 3 hours. The developed fluorescence was detected at different times transferring 20μl of the reaction mixtures in an opportune microplate and adding 280 μl of 0.25 M glycine, pH 10.7. The fluorescence was detected by a Victor microplate reader (Perkin Elmer). Any released enzymatic activity was not measured.

MEDIUM ASSOCIATED β-Hex ACTIVITY

β-hexosaminidase activity associated with the medium was determined on the fluorogenic substrate MUG. Cells were incubated for 2 days in DMEM without phenol red. Aliquots of medium were transferred in a 96-well microplate to perform the enzymatic assays in triplicate. MUG was solubilized at a final concentration of 3 mM in McIlvaine Buffer (0.1 M Citrate/0.2 M Phosphate) pH 5.0. The reaction mixtures were incubated at 37°C under gently shaking for 3 hours. The developed fluorescence was detected at different times transferring 20μl of the reaction mixtures in an opportune microplate and adding 280 μl of 0.25 M glycine, pH 10.7. The fluorescence was detected by a Victor microplate reader (Perkin Elmer). Any released enzymatic activity was not measured.

NEU3 ACTIVITY ASSAY

Neu3 enzyme activity was evaluated using GM3 as substrate. 20 μl of cellular homogenate were incubated with Na-acetate buffer 100mM, pH 4.5, Triton X100 0,04%, 6 μM [^{3}H]GM3, final volume 50 μl. Final concentration of GM3 in micelles is 6 μM containing 5000 dpm of [^{3}H]GM3 labeled with [1-^{3}H] Sphingosine. Buffer and homogenate incubation is made at 37°C under shaking (800 rpm) for 2 hours. at the end of the incubation the reaction is blocked with 1,5 ml chloroform/methanol.

ENZIMATIC ACTIVITIES IN CELL HOMOGENATE

ST ACTIVITY ASSAY

ST-I activity was assayed on neuronal cell homogenates using [3-^{3}H(*sphingosine*)]LacCer as substrate(*228*). ST-II and ST-IV activities were measured as previously described (*231*) using [3-^{3}H(*sphingosine*)]GM3 and [3-^{3}H(*sphingosine*)]GM1 as substrates. The enzymatic reactions of different sialyltransferases were stopped by adding chloroform/methanol (2:1) and analyzed by HPTLC using the solvent system 55:45:10 chloroform/methanol/0.2% aqueous $CaCl_2$. Separated radioactive lipids were detected and quantified by radioactivity imaging performed with a Beta-Imager 2000 instrument (Biospace, Paris, France) and the radioactivity associated with individual lipids was determined with the specific β-Vision software provided by Biospace.

SIALIDASE ACTIVITY ASSAY

Total sialidase activity was determined on neuronal cell homogenates with 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate as a substrate (*201*).
Assays were performed in triplicate. Negative controls for all the assays were performed using heat-inactivated cell homogenates (100°C for 3 minutes). Data were expressed as pmoles of converted substrate/10^6 cells x h and are the means ± SD of three independent experiments.

β-Gal AND β-Glc ACTIVITY ASSAY

In neurons β-galactosidase and β-glucosidase activities associated with total cell homogenates were determined on the fluorogenic substrates: MUB-Gal and MUB-Glc. Briefly, cells were washed twice with PBS, harvested and resuspended in water for the β-glucosidase assay, and in 0.2% Triton X-100 for the β-galactosidase assay. Aliquots of cell homogenate were transferred in a 96-well microplate to perform the enzymatic assays in triplicate. MUB-Gal was solubilized at a final concentration of 0.25 mM in McIlvaine Buffer (0.1 M Citrate/0.2 M Phosphate) pH 4.0, whereas MUB-Glc was prepared at the final concentration of 6 mM in the same buffer at pH 5.2. The reaction mixtures were incubated at 37°C under gently shaking. The developed fluorescence was detected at different times transferring 20μl of the reaction mixtures in an opportune microplate and adding 280 μl of 0.25 M glycine, pH 10.7. The fluorescence was detected by a Victor microplate reader (Perkin Elmer). Data were expressed as pmoles of converted substrate/10^6 cell x h and are the means ± SD of three independent experiments.

MODIFICATION OF THE ACTIVITY OF PROTON PUMPS USING EIPA

The activity of the proton pumps was modified using EIPA at two different concentrations (0,1 μM and 1 μM). EIPA was solubilized in DMEM without phenol red medium at pH 7.3. Cells were pre-incubated at room temperature for 30 minutes and then we performed the different enzymatic activity assay on the PM as above with the only difference of the presence of EIPA also during the assay.

CELL VIABILITY DETERMINATION

The cell viability and membrane integrity of granular cells used for cell surface enzymatic assays were determined using both AM-Calcein staining and Trypan-blue exclusion method. For the calcein staining cells were washed with PBS, the plate was then put on ice and 200 μl of calcein (6.25 μg/ml in PBS) were added to each well. Cells were incubated for 15 minutes at 37°C, 5% CO2. Calcein is then removed and to each well 100 μl of PBS and 50 μl of 1%

Triton X-100 were added. Plate was stirred at room temperature for 15 minutes and then the fluorescence was detected by a Victor microplate reader (Perkin Elmer).

SDS-ZYMOGRAPHY

Culture media were thawed in ice and mixed 3:1 with sample buffer (containing 10% SDS). Samples (15 μg of total protein per sample) were run under non-reducing conditions without heat denaturation onto 10% polyacrylamide gel (SDS-PAGE) co-polymerized with 1 mg/ml of type I gelatin. The gels were run at 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each and incubated overnight in a substrate buffer at 37°C (Tris-HCl 50 mM, $CaCl_2$ 5 mM, NaN_3 0.02%, pH 7.5). The matrix metalloproteinase (MMP) gelatinolytic activity was detected after staining the gels with Coomassie brilliant blue R250, as clear bands on a blue background. Lysis bands were quantified by densitometric scanning (UV Band, Eppendorf).

CELL LIPIDS ANALYSIS

Neuronal granule cells (2^{nd}, 8^{th} and at the 17^{th} day in culture) were incubated in the presence of 3×10^{-8} M [1-^{3}H] Sphingosine. After 2 hours of pulse the medium was removed and replaced with fresh medium without radioactive sphingosine for fibroblasts and with cell-conditionated medium for granule cells. Then, cells were incubated for 48 h of chase. Under these conditions, free radioactive sphingosine was hardly detectable in the cells, and all sphingolipids and phosphatidylethanolamine (obtained by recycling of radioactive ethanolamine formed in the catabolism of [1-3H]sphingosine) were metabolically radiolabeled (*232, 233*).

Cells were harvested in ice-cold water (2 ml) by scraping with a rubber policeman, and lyophilized. Lipids were extracted twice with chloroform/methanol/water 2:1:01 by volume (first extraction, 1.5 ml; second extraction, 0.25 ml). Lipids were separated by monodimensional HPTLC carried out with the solvent systems chloroform/methanol/0.2% aqueous CaCl2, 50:42:11 or 55:45:10 (v/v). The identity of radioactive lipids was assessed by comparison with standard lipids.

WESTERN BLOT ANALYSIS

The same amount of proteins derived from the cell lysates obtained from neural granule cells at different days were analysed by the SDS-PAGE and, after separation, proteins were transferred to PVDF membranes. The presence of MAP2, TAU, Tag-1, L1, Synapsin, Neuroglycan C and PSD95 was assessed using the specific primary antibody antibodies and the opportune horseradish peroxidase-conjugate secondary antibodies. β-actin and β-tubulin were used as loading control. The antibody staining was followed by enhanced chemiluminescence detection (PierceSupersignal). The data acquisition was performed using a GS-700 Imaging Densitometer (Bio-Rad).

OTHER ANALYTICAL METHODS

The radioactivity associated with lipids was determined by liquid scintillation counting. Digital autoradiography of the HPTLC plates was performed with a Beta-Imager 2000 instrument (Biospace, Paris) using an acquisition time of about 24 hours. The radioactivity associated with individual lipids was determined with the specific β-Vision software provided by Biospace. Protein content was determined according to Lowry (*234*) using bovine serum albumin as reference standard.

RESULTS

EVALUATION OF THE PM-ASSOCIATED ENZYME ACTIVITY

Previous studies have demonstrated the presence at PM level of glycohydrolases involved in a complex network of metabolic pathways (*1, 3, 235*). Therefore, it was decided to set up a new method simpler respect the ones used in the previous experiments.

Using human fibroblasts, it was set up an HTA (high throughput assay) method able to measure the activity of β-galactosidase, CBE sensitive β-glucosidase, GBA2 β-glucosidase, SMase and β-hexosaminidases, enzymes working directly at the plasma membrane level using MUB derivates as substrate (Table 1) The substrates were solubilized in cell culture medium at pH 7.3 without serum and phenol red and then added to a fibroblasts monolayer.

In order to discriminate between the CBE-sensitive β-glucosidase and the β-glucosidase GBA2, cells were pre-treated with 5nM *N-(*5.adamantane-1-yl-methoxy)pentyl-deoxynojirimycin or 1 μM CBE respectively for 30 minutes at room temperature and then the enzymatic assays were performed as well in presence of these inhibitors.

After 30 minutes up to 6 hours of incubation at 37°C the fluorescence associated to the cell and the medium was detected by fluorimeter (Figure 19).

ENZYME	ARTIFICIAL SUBSTRATE
Sphingomyelinase (SMase)	-Hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (HMU-PC)
β-Galactosidase (β-Gal)	4-Methylumbelliferyl-β-D-galactopyranoside (MUB-Gal)
CBE-sensitive β-glucosidase	4-Methylumbelliferyl-β-D-glucopyranoside (MUB-Glc) + *N*-(5-adamantane-1-yl-methoxy)pentyldeoxynojirimycin (AMP-DNM)
CBE insensitive β-glucocerebrosidase GBA2	4-Methylumbelliferyl-β-D-glucopyranoside (MUB-Glc) + Conduritol B Epoxide (CBE)
Hexosaminidase (β-Hex)	4-Methylumbelliferyl-β-N-acetylglucosaminide (MUG)
Hexosaminidase A (β-Hex A)	4- Methylumbelliferyl-β-N-acetylglucosaminide-6-sulphate (MUGS)

Tab. 1: identification of the appropriate artificial substrate used to evaluate the activity of each enzyme.

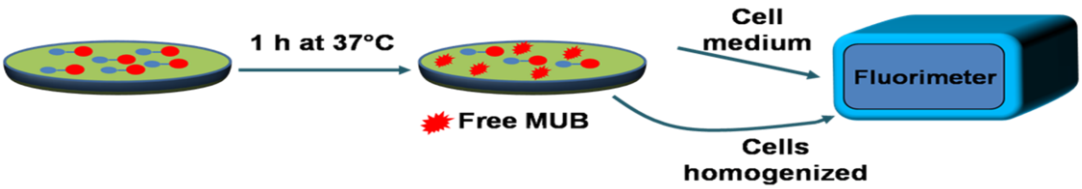


Fig. 19: High throughput assay method able to measure the activity of β-galactosidase, CBE sensitive β-glucosidase, GBA2 β-glucosidase, SMase and β-hehosaminidase only at the plasma membrane level using MUB derivates as substrate

It was verified that even in this situation the HTA method provides information on the activities of only plasma membrane associated enzymes (Table 2).

ENZYME	ACTIVITY
SMase	579,955 ± 4,794 pmoli/(106cells*h)
β-Galactosidase	132,11 ± 32,427 pmoli/(106cells*h)
CBE-sensitive β-glucosidase	2730 ± 129,583 pmoli/(106cells*h)
BE insensitive β-glucocerebrosidase GBA2	515,5 ± 29,817 pmoli/(106cells*h)
Hexosaminidase	47,625 ± 6,168 nmoli/(106cells*h)
Hexosaminidase A	7,528 ± 2,265 nmoli/(106cells*h)

Tab. 2: evaluation of the activity of each enzyme.

Besides, it was proved that the fluorigenic substrates does not have intracellular catabolism because no fluorescence has been found in the cell lysate of fibroblasts on such as the HTS was performed. In addition, it was verified that there is not an unusual fusion of lysosomes with the plasma membrane as a result of changes in extracellular pH as described on human breast cancer cells (*236*). Cells were incubated over night in their media (at different pH, as described above) and an experiment of enzymatic activity was subsequently conduced using each substrate described above: it was obtained as result the lack of enzymatic activity and this clearly highlights the fact that there is not any activity released (data not shown).

Moreover, in order to study more thoroughly the matter of pH dependent lysosomal fusion with the PM, using the same media in which released activity were verified, zymography experiments were lead. This experiment showed that in these cells, at HTA experimental conditions, is not observed any increased release of MMP2 (a typical matrix metalloproteinase released in the extracellular environment by the lysosomal fusion with the PM) in no one of the tested media. This indicates, on one hand, the maintenance of constant lysosomal fusion, on the other, that this fusion not influenced by the extracellular pH.

Therefore we started to characterize the kinetic parameters evaluating the best pH conditions to obtain the maximum activity of each enzymes using the appropriate artificial substrate using as cellular model human fibroblast in culture.

The optimum pH working condition of each enzyme was established performing a HTA assay using solubilized substrates in cell cultured medium without serum and phenol red at different pH:

- ✓ 5.10
- ✓ 5.45
- ✓ 5.70
- ✓ 6.20
- ✓ 7.30
- ✓ 7.90
- ✓ 8.40

Thus we determined the optimum pH condition for each enzyme: SMase (Figure 20), β-galactosidase (Figure 21), β-hexosaminidase (Figure 24) and β-hexosaminidase A (Figure 25) show the maximum activity at pH 5.10 ,whereas CBE sensitive β-glucosidase at pH 6.20 (Figure 22) and GBA2 β-glucosidase at pH 5.7 (Figure 23).

By calcein staining and Trypan blue exclusion method it was found that cells at different pH had the same proliferation and viability .

The data are expressed as pmoles/(10^6 cells *x* h) the date are strictly associated to the substrate concentration for this reason the activity are relative and not absolute. Data are the mean ± SD of three different experiments. Statistical significance of differences was determined by Student's *t-test* and Two-way *ANOVA*. *$p < 0.005$ of the activity measured versus the activity measured at different pH

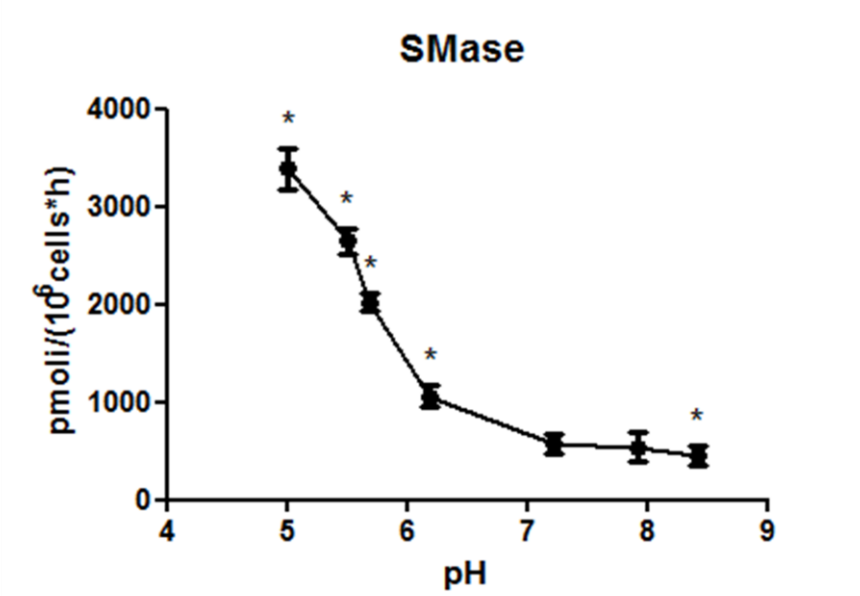


Fig. 20: variation of PM-associated SMase activity at different pH conditions

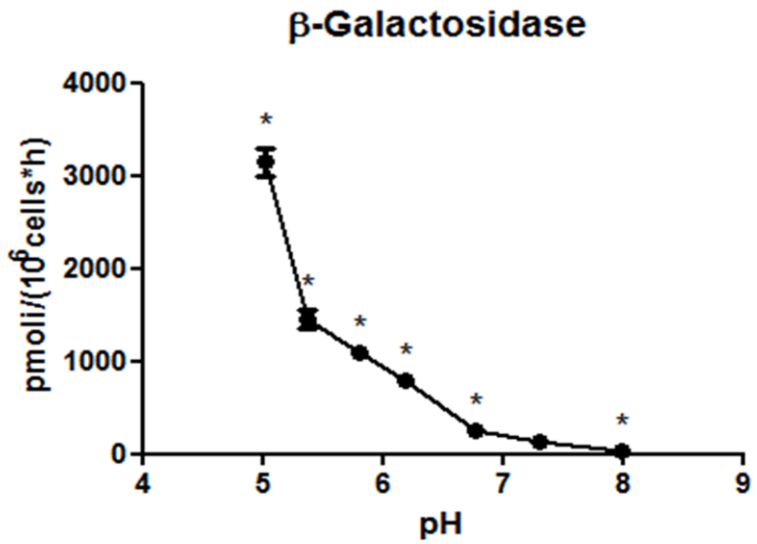


Fig. 21: variation of PM-associated β-galactosidase activity at different pH conditions

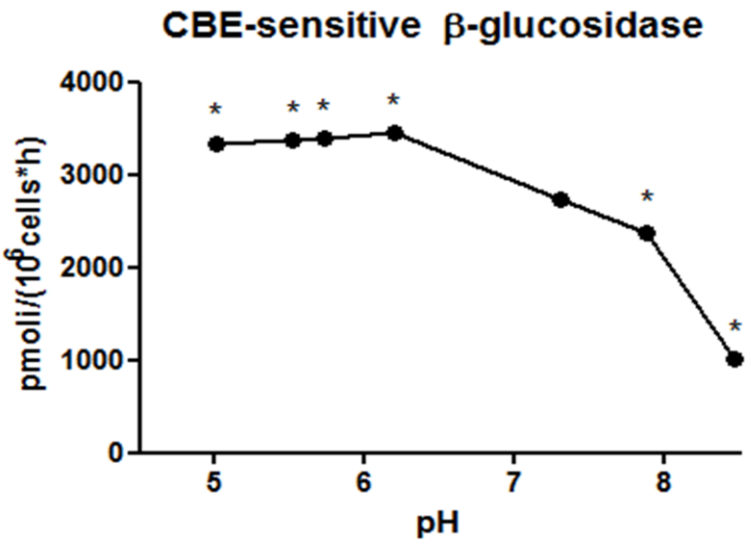


Fig. 22: variation of PM-associated CBE-sensitive β-glucosidase activity at different pH conditions

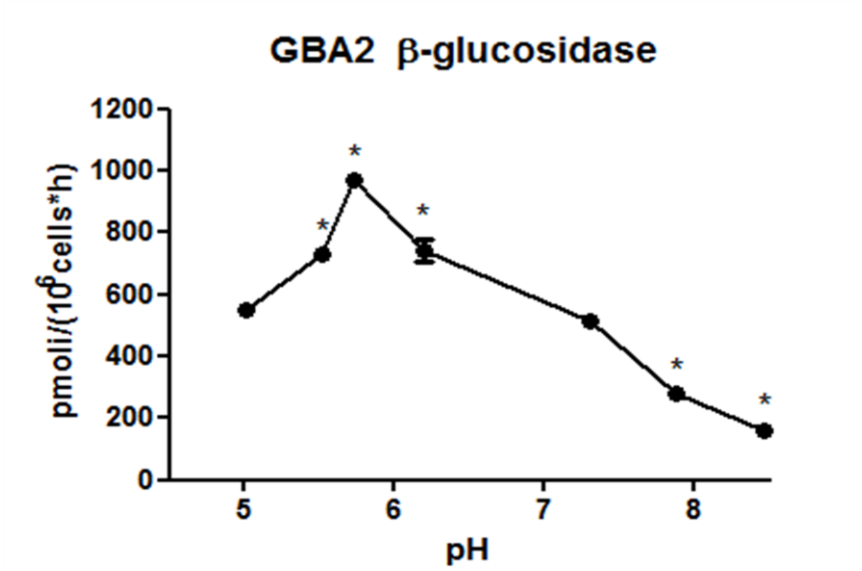


Fig. 23: variation of PM-associated GBA2 β-glucosidase activity at different pH conditions

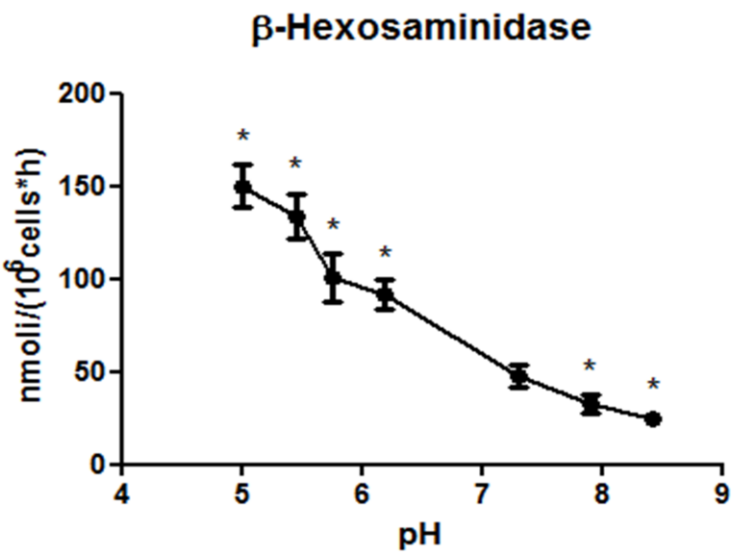


Fig. 24: variation of PM-associated β-hexosaminidase activity at different pH conditions

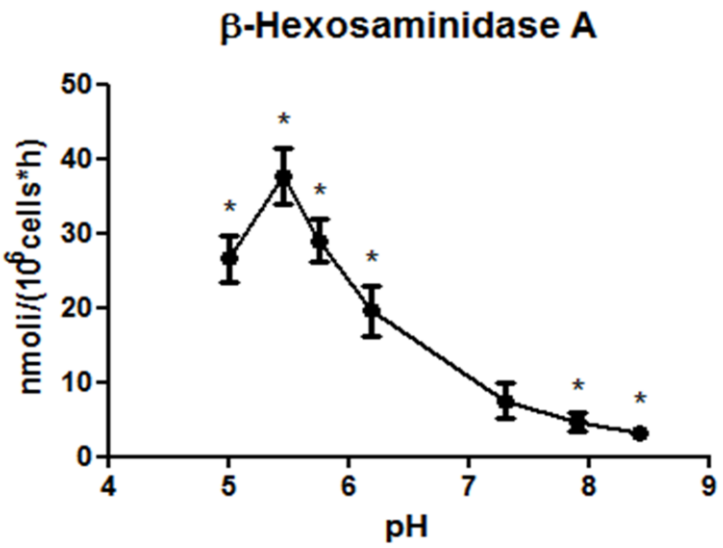


Fig. 25: variation of PM-associated β-hexosaminidase A activity at different pH conditions

DETERMINATION OF Vmax AND Km DIRECTLY ON EXTERNAL LEAFLET OF THE PM OF LIVING CELLS

After we estabilished the optimum of pH of each enzyme we were able to determine the Km and the Vmax of the CBE-sensitive β-glucosidase (Figure 26) and GBA2 β-glucosidase (Figure 27).

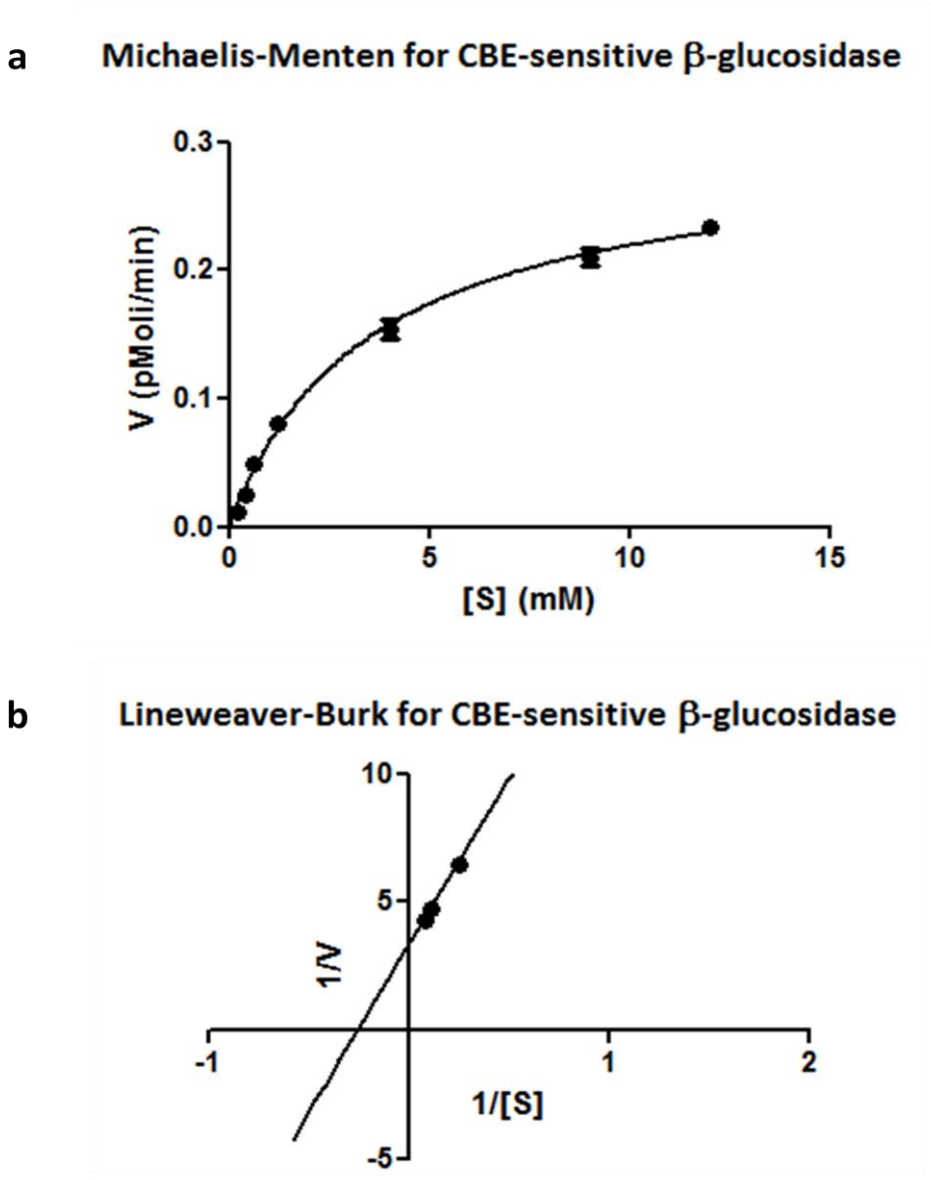
CBE-sensitive β-glucosidase

The experiment was carried out evaluating the reaction at growing substrate concentration. As it can be seen the kinetics has an hyperbolic trend (Figure 26a) and it was possible to calculate the value of Km (3,313 mM) and Vmax (0,2965 pmol/min) by Lineweaver-Burk graph (Figure 26b).

GBA2 β-glucosidase

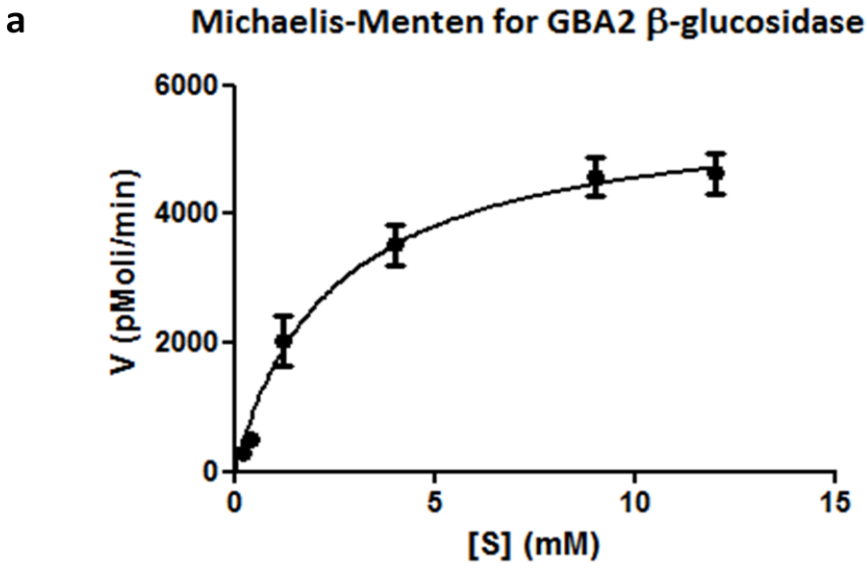
The experiment was carried out evaluating the reaction at growing substrate concentration. As it can be seen the kinetics has an hyperbolic trend (Figure 27a) and it was possible to calculate the value of Km (2,435 mM) and Vmax (0,05560 pmol/min) by Lineweaver-Burk graph (Figure 27b).

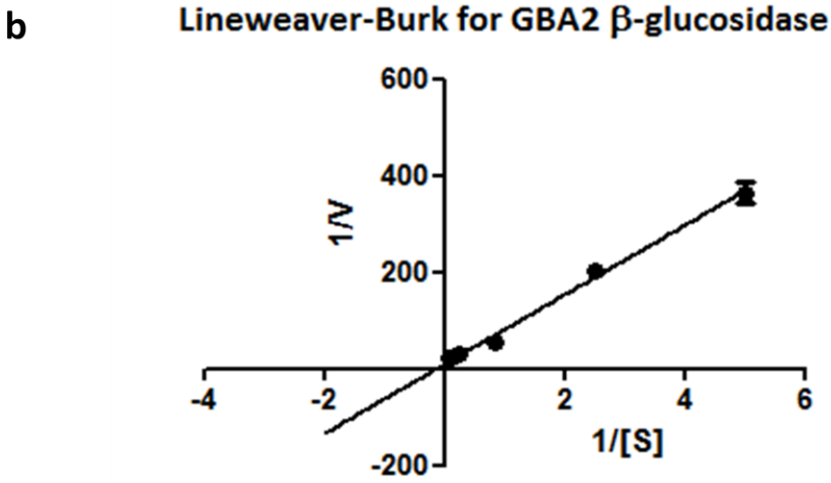
At the end of the experiments we evaluated by calcein staining and Trypan blue exclusion method, it was evaluated that cells in both experiments had the same proliferation and viability indicating the absence of substrate toxicity.



Km = 3,313 ± 0,3000 mM
Vmax = 0,2965 ± 0,009158 pmol/min

Fig. 26: Km and Vmax of CBE-sensitive β-glucosidase evaluated directly on the external leaflet of the PM of living fibroblasts





Km = 2,435 ± 0,2132 mM
Vmax = 0,05560 ± 0,001605 pmol/min

Fig. 27: Km and Vmax of GBA2 β-glucosidase evaluated directly on the external leaflet of the PM of living fibroblasts

DETERMINATION OF ENZYME ACTIVITY AFTER ADMINISTRATION OF NHEs INHIBITOR (EIPA)

In the previous paragraph it was found that the plasma membrane associated enzymes shows acidic pH as best pH working conditions, this can be considered strange being this

enzymes associated to the plasma membrane whose pH is usually defined neutral. However in the plasma membrane there are different proton pump that could locally shift the neutral pH to acidic pH. In fact at least 7 different families of transporters/exchangers are present at this level and are capable of varying the pH locally. In order to verify whether the varying of the membrane potential acting on these pumps can influence also the activity of these enzymes, we carried out preliminary experiments using 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), an inhibitor of NHEs (Na^+/H^+) exchangers solubilized together with appropriate fluorigenic substrates in cell culture medium without phenol red and serum at pH 7.4. As control we used cells without EIPA incubation.

We determined the activity of β-galactosidase (Figure 28), CBE sensitive β-glucosidase (Figure 29), GBA2 β-glucosidase (Figure 30), β-hexosaminidase (Figure 31) and β-hexosaminidase A (Figure 32) after administration of EIPA at two different concentrations:

- ✓ 0,1 μM
- ✓ 1 μM

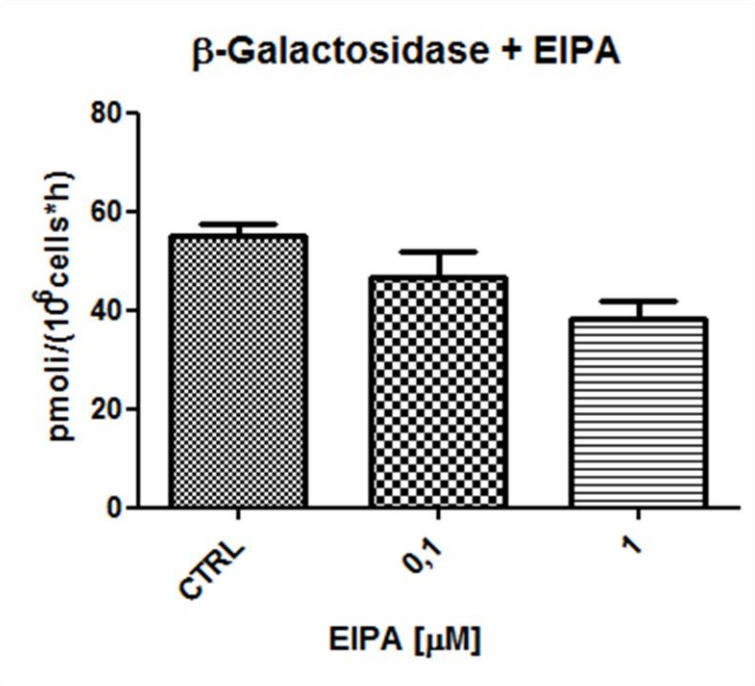


Fig. 28: variation of PM-associated β-galactosidase activity after EIPA administration

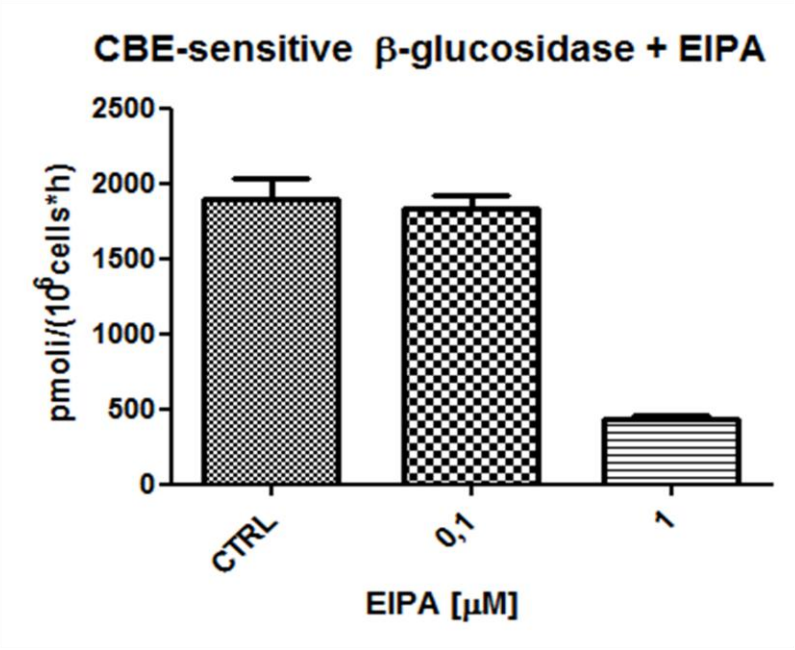


Fig. 29: variation of PM-associated CBE-sensitive β-glucosidase activity after EIPA administration

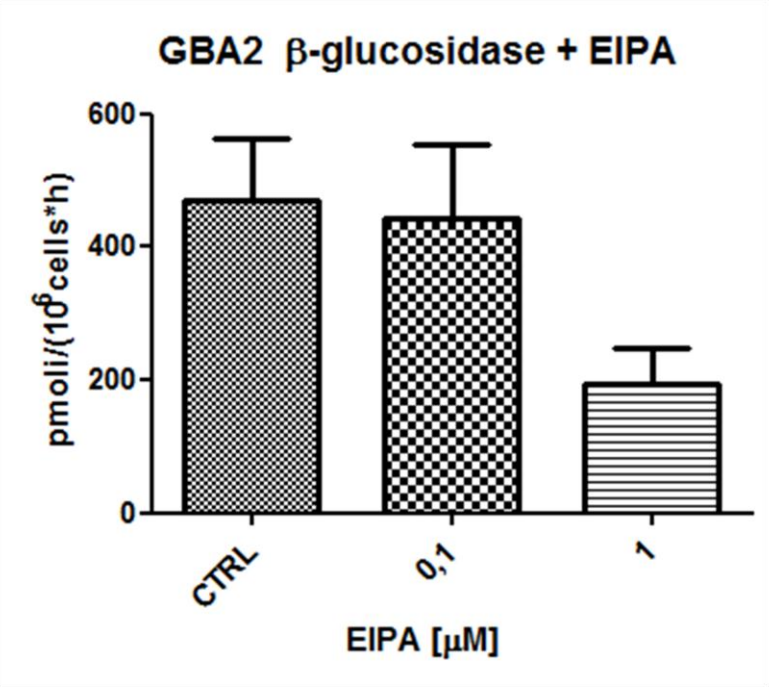


Fig. 30: variation of PM-associated GBA2 β-glucosidase activity after EIPA administration

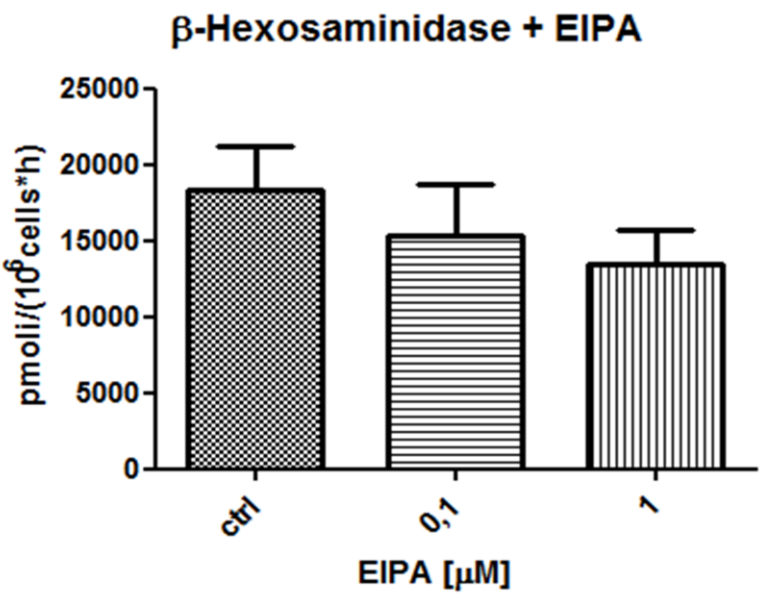


Fig. 31: variation of PM-associated β-hexosaminidase activity after EIPA administration

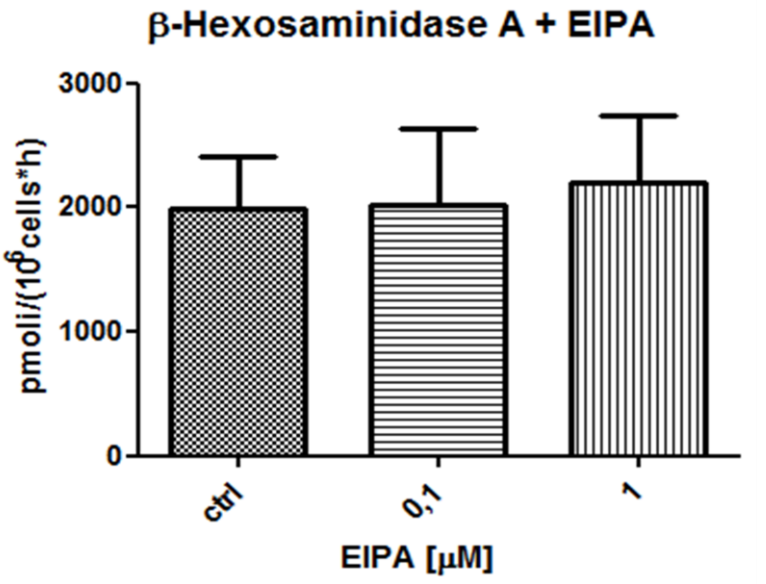


Fig. 32: variation of PM-associated β-hexosaminidase A activity after EIPA administration

As it can be seen in the graphs EIPA 0,1 μM doesn't affect the activity of the enzyme.

EIPA 1 μM contrariwise, on the one hand is able to decrease the activity of β-galactosidase (Figure 28), CBE sensitive β-glucosidase (Figure 29) and GBA2 β-glucosidase (Figure 30), on

the other it doesn't have any effect on the activity of β-hexosaminidase (Figure 31) and β-hexosaminidase A (Figure 32). This could be due to a different localization of these two enzymes on the plasma membrane respect to the others above. So, EIPA 1 μM seems to be able to modulate the activity of NHEs (Na^+/H^+) exchangers present in some areas of the PM with the following variation of the pH and the modulation of the enzyme activities.

At the end of the experiment it was evaluated, by calcein staining and Trypan blue exclusion method, that cells in all experiments had the same proliferation and viability indicating that EIPA is not toxic.

The data are expressed as pmoles/(10^6 cells *x* h) and are the mean ± SD of three different experiments.

GLYCOHYDROLASES ACTIVITY DURING IN VITRO NEURONAL DIFFERENTIATION OF RAT CEREBELLAR GRANULE CELLS

After a partially characterization of these PM-associated glycohydrolases we start to investigate about their functional role during the cell live and in particular we focused our attention to study their involvement during the neuronal differentiation. As well known from the literature during the neuronal differentiation the glycosphingolipid content and pattern underwent to a strong remodelling. For these reasons we think that this could be a good starting point to study the involvement of the PM glycohydrolases in cell physiology.

CHARACTERIZATION OF THE RAT CEREBELLAR GRANULE CELLS DEVELOPMENT *in vitro*

Cerebellar granule cells were prepared from 8-day-old Sprague–Dawley rats, and allowed to differentiate *in vitro.* These cells immediately start to differentiate and spontaneously undergo a developmental pattern that resembles the one of cerebellar neurons *in vivo*, but in a condensed time scale, reaching fully differentiated state after 8 days. During *in vitro* development it was observed the formation of an important net of neurites and the establishment of synapses which are made possible by the production of new specialized membrane, in fact all these events are accompanied by dramatic changes in the lipid content and pattern (*56*).

Glycerophospholipids and cholesterol, that represent the bulk of membrane lipids, significantly increase within the days in culture, maintaining however a very similar pattern during cell differentiation (data not shown).

Sphingolipid content underwent a marked increase during neuronal development. Along the days in culture, cell morphology (Figure 33a), expression of specific proteins (Figure 33b), and sphingolipid patterns (Figure 33c) were determined to confirm neuronal development and aging.

Rat cerebellar granule cells at the 2nd day in culture already emit short neurite-like processes. At the 8th day in culture cells are mostly grouped in large aggregates connected by an important net of neurites. This morphology is emphasized with the cell aging and, at the 17th day in culture, the cell bodies are organized in very big clumps connected by bundles of fasciculate neuritis (Figure 33a).

The microtubule associated protein MAP2, a neuronal marker typically associated to dendrites, is present in the isoforms of 80 and 280 kDa (*237-239*) and its content reached the maximum level between the 7th and 9th day in culture, whereas it is possible to observe a down–regulation in aged cells. A similar expression pattern could be observed for low molecular weight Tau proteins, a microtubule associated proteins which promote process formation in neurons and are preferentially targeted in axons of the CNS neurons (*239, 240*).

As already reported for other neuronal cell cultures, TAG-1, a glycoprotein responsible for the axon extension, showed the maximum expression at the earliest stages of differentiation and gradually reduced during the days in culture. On the other hand, L1 that is involved in the onset of axon fasciculation (observed in these cells after the 8th day in culture) showed an opposite behavior, reaching the maximum expression during the cell aging (*241-245*). Synapsin, Neuroglycan C and Post Synaptic Density protein 95 (PSD95) are typical synaptic markers (*246-250*). Their expression in rat cerebellar granule cells appeared very similar, being almost undetectable at the first days in culture whereas their content increase from the 7th day in culture and remained high even in senescent neurons (Figure 33b).

The analysis of the sphingolipid pattern was performed on the total cell lysates obtained from cells at the 2nd, 8th and 17th day in culture by steady-state metabolic labeling using [1-3H]sphingosine. Data obtained (Figure 33c) were in accordance to those already published (*56*).

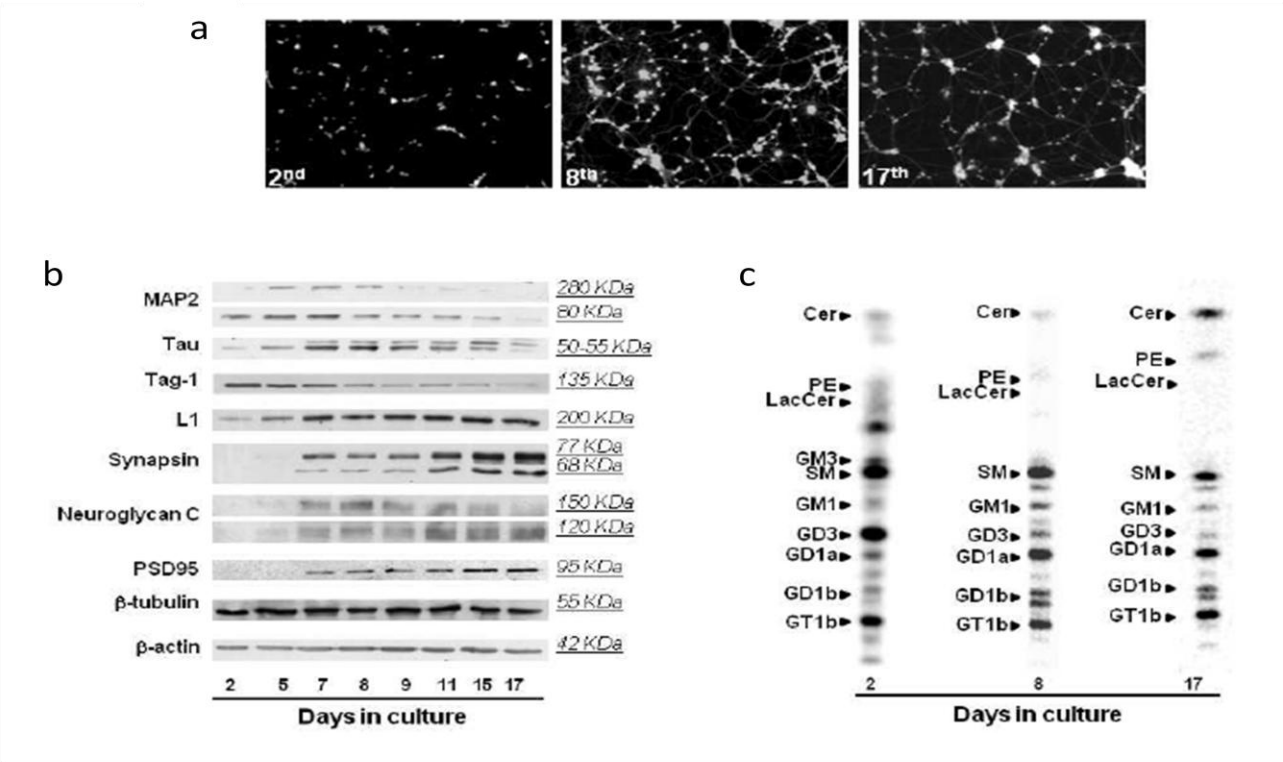


Fig. 33: **a**. Immunofluorescence acquisition of rat cerebellar granule cells at the 2nd, 8th and 17th day in culture stained with calcein. **b**. Similar amounts of proteins from cells at different days in culture were loaded on SDS-PAGE and then transferred on a PVDF membrane followed by detection by Western Blotting using specific antibodies for different neuronal marker proteins. **c**. Analysis of radioactive lipids from total cell lysates obtained from rat cerebellar granule cells at the 2nd, 8th and 17th day in culture.

ENZYMATIC ACTIVITIES IN TOTAL CELL HOMOGENATE

The activities of the sialyltransferases ST-I, ST-II and ST-IV, were evaluated in the total cell lysates of granular cells at different day in culture. In agreement with previous data, obtained from human brain development (*54, 251*), it was found that the activities of ST-I and ST-IV increased during the neuronal differentiation. In addition, also the activity of ST-II increased in an *in vitro* assay (Figure 34).

The up-regulated ST-I activity did not produce an increase of the GM3 content. This is probably due to the rapid synthesis of more complex gangliosides from GM3 by ST-II and ST-IV. ST-II acts on the GM3 to produce GD3 whose endogenous content, referred to the cell number, increases 4 fold during cell differentiation even if the highest percentage of radioactive GD3 is at the 2nd day in culture. The absence of correlation between endogenous content and the radioactivity incorporation for GM3 and GD3 at the 2nd day in culture is due to the rapid turnover of these species at the first stages of neural differentiation (*56*). At the same time the increased activities of UDP-GalNac: LacCer/GM3/GD3/GT3 β1-4 *N*-

acetylgalactosaminyltransferase and UDP-galactose: GA1/GM1/GD1b/GT1c β1-4 galactosyltransferase (*252*) subtract GM3 for the synthesis of GM1 and ST-IV allows the synthesis of GD1a from GM1. The same enzymes can lead to the gangliosides of the b-series from GD3.

During cell aging the total cell ganglioside content does not change, while the sialyltransferase activities underwent to a decrease, particularly ST-I and ST-IV. This could be explained by the strong reduction in the gangliosides turnover in senescent neurons (*56*).

Figure 35 shows the activities in total cell homogenates of sialidase, β-galactosidase and β-glucosidase. The sialidase activity in total cell homogenate is mainly due to the activity of the lysosomal sialidase Neu1, whose activity strongly increased during neuronal differentiation whereas it did not significantly change in neuronal aging. A similar trend was observed for the activity of total cell β-glucosidase. Several papers reported about the role of this enzyme in the regulation of the number of axonal branches and length of axonal plexus (*253*). The activity of total β-galactosidase did not increase during neuronal differentiation whereas it is possible to observe a 4 fold increase during the cell aging. This is in agreement with what already presented (*102*) on the increased activity of a "senescence-associated β-galactosidase" in aging hippocampal neurons.

The data are expressed as pmoles/(10^6 cells *x* h) and are the mean ± SD of three different experiments. Statistical significance of differences was determined by Student's t-test and Two-way *ANOVA.* *$p < 0.005$ of the 8th day activity versus the activity measured at the 2nd day in culture. $^{\#}p<0.005$ of the activity measured at the 17th day in culture versus that measured at the 8^{th} day.

Fig. 34: Enzymatic activities in total cell homogenate. Total cell homogenates from rat cerebellar granule cells at three different stages of in vitro development (2nd, 8th and 17th day in culture), were submitted to different enzymatic assays as described under "Materials and Methods". Activities of ST-I, ST-II and ST-IV measured on the natural radioactive substrates.

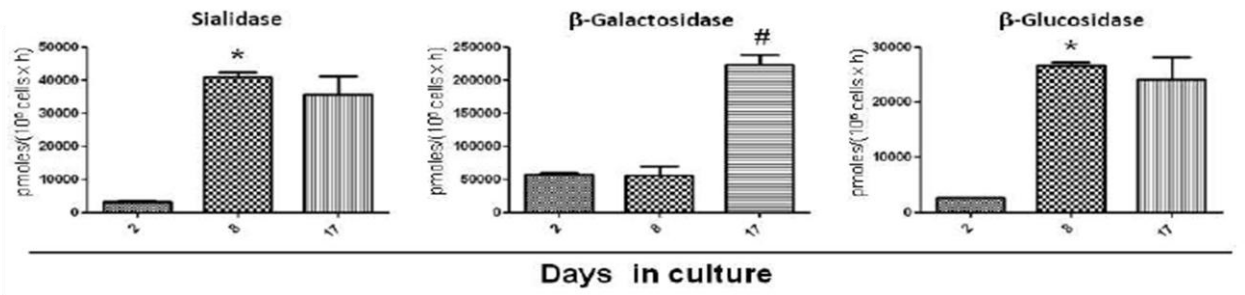


Fig. 35: Enzymatic activities in total cell homogenate. Total cell homogenates from rat cerebellar granule cells at three different stages of in vitro development (2nd, 8th and 17th day in culture), were submitted to different enzymatic assays as described under "Materials and Methods". Activities of total cell sialidase, β-galactosidase and β-glucosidase measured on the fluorogenic artificial substrates.

ENZYMATIC ACTIVITIES AT THE CELL SURFACE

Behind to the evaluation of the total cell activity of the principal glycohydrolases involved in the glycosphingolipid catabolism it was measured, using HTA, also the PM-associated activity during the rat granular cells in vitro differentiation. Each enzymatic activity was performed directly using optimum pH working condition (pH 5.10 for β-galactosidase, pH 5.7 for GBA2 β-glucosidase and pH 6.20 for CBE sensitive β-glucosidase). By calcein staining and Trypan blue exclusion method it was found that the treatments were no toxic for cells. It was found that sialidase Neu3 (Figure 36) reached the maximum activity at day 8th in culture

corresponding to the fully differentiated neuronal stage, and partially decreased during aging. As shown in figure 37, along cell differentiation in culture, it was measured β-galactosidase (Figure 37a), CBE-sensitive β-glucosidase and non-lysosomal β-glucosidase GBA2 activities (Figure 37b). As expected, the PM-associated activity of these enzymes was very low with respect to the corresponding activity found in the total cell homogenates. Differently to what observed for the total β-galactosidase, whose activity did not change along cell differentiation in culture, and increased 4 fold during aging, the β-galactosidase activity associated with the cell surface underwent to a gradual and strong (20 fold from the 2^{nd} to the 17^{th} day in culture) increase during both differentiation and aging. These results can suggest that both total cell β-galactosidase and PM β-galactosidase could be considered markers of senescence. The β-glucosidase activity at the cell surface increased during the different days in culture and this was mainly due to the non-lysosomal β-glucosidase GBA2 that reached a peak of activity during the cell aging (Figure 37b).

By calcein staining and Trypan blue exclusion method it was found that 90-95% of cells used for the cell surface enzymatic assays were alive and the membrane integrity were maintained.

In order to discriminate between the CBE-sensitive β-glucosidase and the non-lysosomal β-glucosidase GBA2, cells were pre-treated with 5nM *N-(*5.adamantane-1-yl-methoxy)pentyl-deoxynojirimycin or 1 μM CBE respectively for 30 minutes at room temperature and then the enzymatic assays were performed as well in presence of these inhibitors. The data are expressed as pmoles/(10^6 cells *x* h) and are the mean ± SD of three different experiments. Statistical significance of differences was determined by Student's *t-test* and Two-way *ANOVA.* *$p < 0.005$ of the activity measured versus the activity measured the previous day.

Fig. 36: Enzymatic activities at the cell surface. The glycohydrolytic activity of Neu3 associated to the cell surface was analyzed in rat cerebellar granule cells at different days in culture and expressed as moles/10^6 cells x h. The activity of PM associated sialidase Neu3 was evaluated on its natural radioactive substrate GM3 as described under "Materials and Methods".

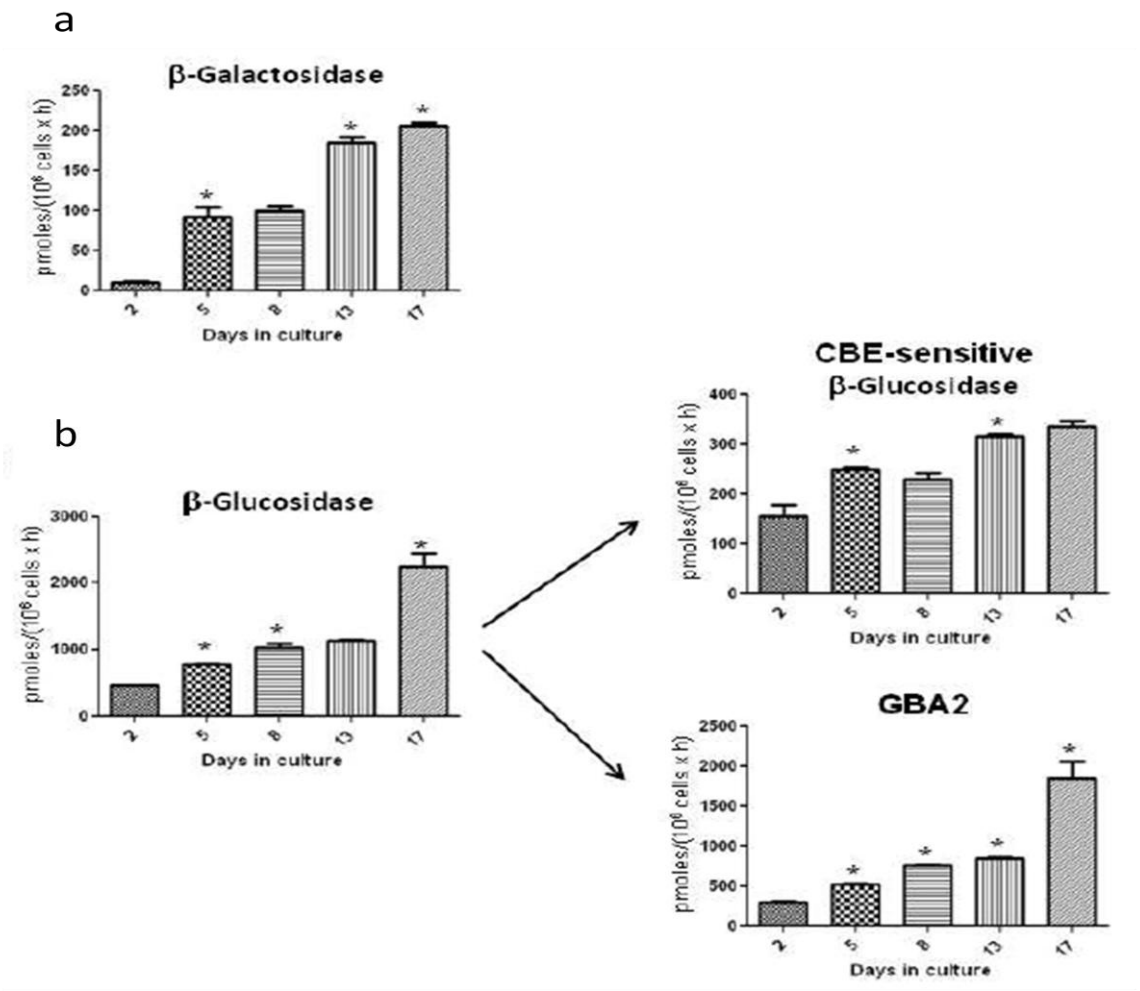


Fig. 37: Enzymatic activities at the cell surface. The glycohydrolytic activities of β-galactosidase and β-glucosidase associated to the cellsurface were analyzed in rat cerebellar granule cells at different days in culture and were expressed as pmoles/10^6 cells x h. The activity of cell surface β-galactosidase was measured using the fluorogenic substrate MUB-Gal directly on living cells, as reported in "Materials and Methods". The same experimental approach has been used for the evaluation of the β-glucosidase activity associated to the PM, using the fluorogenic substrates MUB-Glc.

DISCUSSION

Glycosphingolipids are components of the cell plasma membranes participating in the organization of lipid domains and modulating several aspects of the signal transduction processes (*254*). Plasma membranes glycosyl hydrolases (*179, 185, 191, 192*) seem to be the natural candidates for modifications of the cell surface glycolipids, taking part to the modulation of the signal transduction processes. Recent studies have demonstrate the presence of glycosyl hydrolases non only intrecellularly but also at plasma membrane level (*1, 3, 185*). Among membrane associated glycosyl hydrolases, the membrane bound sialidase Neu3 (*1*), SMase, β-galctosidase, β-glucosidase (*3*) and β-hexosaminidase (*185*) have been characterized. Neu3, together with plasma membrane-associated β-galactosidase and β-glucosidase, participates to the production of bioactive ceramide at the cell surface of human fibroblasts in culture (*1*). The structure of the plasma membrane-associated β-galactosidase is still unknown; it could be the lysosomal enzyme transferred to the plasma membrane during a cell surface repairing process by fusion of lysosomes with plasma membranes (*221*). Three different SMases are available in eukaryotic cells (*255*): secreted SMase, acid SMase and neutral SMase. Secreted SMase exerts its activity in the extracellular environment. Acid SMase are principally located in the lysosomes and few evidences reports about its association to PM. Neutral sphingomyelinase identify a family of different enzymes that catalyze the hydrolysis of SM into ceramide at pH 7.4 and are Mg^{2+}-dependent enzymes. Two distinct β-glucosidases are associated with the plasma membranes of fibroblasts. One enzyme could derive from lysosomes (CBE sensitive β-glucosidase), but the other is the known as GBA2 β-glucosidase, displaying a structure different from that of lysosomes (*256*).The expression levels of the three plasma membrane-associated enzymes Neu3, β-galactosidase and β-glucosidase are interdependent and affected by those of other enzymes of glycosphingolipid metabolism. As for Neu3, plasma membrane β-galactosidase and β-glucosidase display trans activity in living cells being capable to act on substrates belonging to neighboring membranes with no addition of activator proteins or detergents (*3*). The presence of β-hexosaminidase A protein in the external leaflet of plasma membrane has been also demonstrated in cultured fibroblasts (*185*). The membrane protein shows in vitro enzymatic activity on artificial substrates and on natural glycolipids in the presence of GM2 activator protein. Immunological and biochemical characterization of the membrane-associated β-hexosaminidase A indicated that this enzyme has the same structure of the lysosomal enzyme. As demonstrated by different scientist (*221, 257*) a general mechanism to

repair the plasma membrane torn is represented by the fusion of lysosomes membrane. This event could be the way for all the lysosomal glycolipid-metabolizing enzymes to reach the cell surface where they play an active role in remodeling the glycolipid composition together with specific and different membrane-associated enzymes (*235*).

During my thesis periods, I set up an HTA (high throughput assay) method able to measure the activity of SMase, β-galactosidase, CBE sensitive β-glucosidase, GBA2 β-glucosidase and β-hexosaminidases, enzymes working directly at the plasma membrane level of human fibroblast in culture using MUB derivates as substrate. Afterwards we applied this assays in order to study these PM associated activities in rat cerebellar granule cells along differentiation and aging in culture. Up to now the assay used to mesure the activity of these enzymes was a cell free assay based on the isolation of PM proteins by surface biotinylation followed by streptavidin affinity chromatography. PM associated activities were evaluated by *in vitro* assays using the fractions containing the PM proteins but, recently, we have developed this method capable to evaluate the activity of enzymes involved in the sphingolipids metabolism directly on the plasma membrane of living cells (*in vivo*) (*2*).

So, I set up an high trough put assay for the detection of cell surface SMase, β-galactosidase, β-glucosidase and β-hexosaminidases activities using fluorigenic artificial substrates that are: HMU-PC to detect SMase activity, MUB-Gal for β-galactosidase and MUB-Glc in presence of 5 nM AMP-DNM, that is a specific inhibitor of GBA2 β-glucosidase, in order to detect the lysosomal β-glucosidase activity whereas, to detect GBA2 β-glucosidase activity, we used MUB-Glc in presence of 1 μM CBE, that is a specific inhibitor of CBE sensitive β-glucosidase (Table 1). Finally we used MUG to detect β-hexosaminidase activity and MUGS for β-hexosaminidase A. The substrates were solubilized in cell culture medium without serum and phenol red and then added to a fibroblast monolayer. After one or more hours of incubation at 37°C the fluorescence associated to the cell and to the medium was detected by fluorimeter. No fluorescence was found associated to cell homogenates, this means that artificial substrates do not enter into the cells, whereas we found fluorescence associated to the medium. So, we verified that the fluorescence detected in the cell medium was not due to a release of the enzyme during the assay, so the measured activity was really due to the enzymes present at the cell surface (data not shown).

Using this methods we stating to characterize some characteristic parameter of this enzymes when they work in living cell using human fibroblast as cellular model. First of all I

characterized the best pH working condition for each PM-associated enzyme. SMase shows the highest activity at pH 5.10 with a gradual decrement of the activity from pH 5.4, the activity at neutral and alkaline pH is about 4 times lower than the highest. β-Galactosidase shows also the highest activity at pH 5.10 with a sudden drop of the curve and practically no activity already at alkaline pH. The two isoforms of β-glucosidase show different trends in enzyme activities: on the one hand CBE sensitive β-glucosidase activity is very high at acidic pH (with highest activity at pH 6.2) while it decrease starting from neutral pH and reaching 3 time less value at alkaline pH; on the other GBA2 β-glucosidase shows the highest activity at 5.7 pH (twice than at 5.0 pH) and, after this value, there is a gradual decrease of the activity. Finally β-hexosaminidases presents very high activity at 5.0 pH with gradual decrease of it until three times lower activity at alkaline pH, while β-hexosaminidases A has the same course but with a peak at 5.2 pH.

For the two isoforms of β-glucosidase we performed, working at the best pH conditions, also kinetics experiments in order to determine Km and Vmax parameters. The values founded resulted different from those already published obtained by the cell free assay. This difference is not so surprising because in this assay we evaluated the kinetics parameters of enzyme that residing and working in their natural environment.

So, what we found in this first group of experiments was that the plasma membrane associated enzymes showed acidic pH as best pH working conditions. This can be considerate strange because this enzyme is associated to the plasma membrane whose pH is usually defined neutral. However in the plasma membrane there are different proton pumps that can locally shift the pH from neutral values to acidic ones (*222, 226*). Moreover also the extracellular environment could play an important role. In particular situations, as in the tumor core or during inflammatory events, it is possible to observe an acidification of the cellular environment. We can so speculate that these enzymatic activities could be varied directly by the cells through the modulations of proton pumps or indirectly by phenomena that change the extracellular environment.

For this reason, we set up a second group of experiments using EIPA, the specific inhibitor of NHEs exchangers, in order to verify whether the varying of membrane potential acting on these pumps can influence also the activity of PM-glycohydrolases. For each enzyme we set up the assay using 0,1 μM and 1 μM EIPA versus control. What we can achieve from the graphs (Figure 34-39) is that 0,1 μM EIPA doesn't have any effect on the activity of the

enzymes, this is in accord to data already publisced showing that this concentration is not sufficient for the inhibitions of the NHEs proton pumps present at the plasma membrane level. 1 μM EIPA has, contrariwise, effect on some of the enzymes. We can see a small decrease (30%) of the activity of β-galactosidase, while there is a huge decrease on the activity of both the two β-glucosidase isoforms: the one of CBE sensitive β-glucosidase diminishes of the 71% after the administration of 1 μM EIPA while the one of GBA2 β-glucosidase was reduced of the 59%. Surprisingly, however, the activity of β-hexosaminidase (and β-hexosaminidase A) is nowise influenced by the administration of 1 μM EIPA and this could be related to a different localization of the enzyme on the plasma membrane, where the NHEs exchangers doesn't reside. So, we can speculate that the activity of this enzyme could be regulated by different proton pumps from that one and we are going to try different inhibitors from EIPA to study the action of others exchangers.

After having developed the method of evaluation of PM glycohydrolases we decided to use it in order to evaluate if these plasma membrane associated enzymes could be involved in the neuronal differentiation.

During neuronal development it was possible to observe dramatic changes in the glycosphingolipids content that cause a parallel reorganization of the PM lipid domains enriched in glycosphingolipids (*54, 56, 251*). The driving forces guiding these modifications are not completely understood, however some information regarding the possibility of a fine tuning of cell PM glycolipid composition have already been obtained in other cellular models where it has been observed a synergy among different glycohydrolases associated with the cell surface (*1, 3*). Rat cerebellar granule cells represent a suitable *in vitro* model of neuronal cells as demonstrated by morphological and biochemical approaches. In rat cerebellar granule cells it has been observed an increase of the total cell ceramide content (8 fold from the 2nd to the 17th day in culture) (*56*) and of that belonging to the sphingolipid-enriched domains (10 fold from the 2nd to the 17th day in culture) (*232*), whereas a parallel reduction of the endogenous content of both sphingomyelin and gangliosides was observed in sphingolipid-enriched domains of senescent cells respect to fully differentiated neurons (*56*). The increase of ceramide could thus be explained with the well-known ceramide-sphingomyelin cycle, that has been widely demonstrated to be correlated to the apoptotic phenomena (*258*). Nevertheless the increased activity of the PM associated glycohydrolases during the cell aging (Figure 43), can let speculate that the augmented ceramide in the PM

could also derive from the catabolism of glycosphingolipids directly at the cell surface, as it has been previously demonstrated in human fibroblasts (*1*). As it was reported (*97, 98*), the sialidase Neu3 is able to influence the protrusion and symmetry of axons in neuronal cells probably by a local change of the PM sphingolipid composition at the axonal cones., in my experiments I obtained different activity of Neu3 during the day in culture reaching the maximum activity after the 8 day when the granul cell are morphologically and biochemically fully differentiated neurons. We can thus hypothesize that the modulation of the activities of other PM-associated glycohydrolases during the neuronal differentiation could be functional to the differentiation itself, and could also be useful to define the curvature properties of specific area of the PM (such as the synapses or the negative curvature of the membrane near the axon protrusion) by an *in situ* modification of the glycosphingolipids components. In particular, considering the typical geometry of the synapses, characterized by the succession of PM regions with negative and positive curvatures, that can be correlated respectively to a major presence of simple sphingolipids or to an enrichment of more complex glycosphingolipids (*259*), we could speculate that the balance between glycosylation and de-glycosylation events at the cell surface could be a very important mechanism responsible to establish and maintain this local membrane curvature during the cell life.

REFERENCES

1. Valaperta, R., Chigorno, V., Basso, L., Prinetti, A., Bresciani, R., Preti, A., Miyagi, T., and Sonnino, S. (2006) Plasma membrane production of ceramide from ganglioside GM3 in human fibroblasts, *FASEB J 20*, 1227-1229.
2. Aureli, M., Loberto, N., Lanteri, P., Chigorno, V., Prinetti, A., and Sonnino, S. (2010) Cell surface sphingolipid glycohydrolases in neuronal differentiation and aging in culture, *Journal of Neurochemistry doi: 10.1111/j.1471-4159.2010.07019.x*.
3. Aureli, M., Masilamani, A. P., Illuzzi, G., Loberto, N., Scandroglio, F., Prinetti, A., Chigorno, V., and Sonnino, S. (2009) Activity of plasma membrane beta-galactosidase and beta-glucosidase, *FEBS Lett 583*, 2469-2473.
4. Svennerholm, L., Bostrom, K., Fredman, P., Mansson, J. E., Rosengren, B., and Rynmark, B. M. (1989) Human brain gangliosides: developmental changes from early fetal stage to advanced age, *Biochim Biophys Acta 1005*, 109-117.
5. Yu, R. K., Macala, L. J., Taki, T., Weinfield, H. M., and Yu, F. S. (1988) Developmental changes in ganglioside composition and synthesis in embryonic rat brain, *J Neurochem 50*, 1825-1829.
6. Irwin, L. N., and Irwin, C. C. (1982) Development changes and regional variation in the ganglioside composition of the rat hippocampus, *Brain Res 256*, 481-485.
7. Kotani, M., Terashima, T., and Tai, T. (1995) Developmental changes of ganglioside expressions in postnatal rat cerebellar cortex, *Brain Res 700*, 40-58.
8. Kracun, I., Rosner, H., Drnovsek, V., Vukelic, Z., Cosovic, C., Trbojevic-Cepe, M., and Kubat, M. (1992) Gangliosides in the human brain development and aging, *Neurochem Int 20*, 421-431.
9. Pomorski, T., Hrafnsdottir, S., Devaux, P. F., and van Meer, G. (2001) Lipid distribution and transport across cellular membranes, *Semin Cell Dev Biol 12*, 139-148.
10. Edidin, M. (2003) Lipids on the frontier: a century of cell-membrane bilayers, *Nat Rev Mol Cell Biol 4*, 414-418.
11. Sprong, H., van der Sluijs, P., and van Meer, G. (2001) How proteins move lipids and lipids move proteins, *Nat Rev Mol Cell Biol 2*, 504-513.
12. Mellman, I. (1996) Membranes and sorting, *Curr Opin Cell Biol 8*, 497-498.
13. Prinz, W. (2002) Cholesterol trafficking in the secretory and endocytic systems, *Semin Cell Dev Biol 13*, 197-203.
14. Munro, S. (2003) Lipid rafts: elusive or illusive?, *Cell 115*, 377-388.
15. Schlegel, R. A., and Williamson, P. (2001) Phosphatidylserine, a death knell, *Cell Death Differ 8*, 551-563.
16. Williamson, P., and Schlegel, R. A. (2002) Transbilayer phospholipid movement and the clearance of apoptotic cells, *Biochim Biophys Acta 1585*, 53-63.
17. Feizi, T. (1985) Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens, *Nature 314*, 53-57.
18. Caputto, R., Maccioni, H. J., Arce, A., and Cumar, F. A. (1976) Biosynthesis of brain gangliosides, *Adv Exp Med Biol 71*, 27-44.
19. Partington, C. R., and Daly, J. W. (1979) Effect of gangliosides on adenylate cyclase activity in rat cerebral cortical membranes, *Mol Pharmacol 15*, 484-491.
20. Kim, J. Y., Goldenring, J. R., DeLorenzo, R. J., and Yu, R. K. (1986) Gangliosides inhibit phospholipid-sensitive Ca2+-dependent kinase phosphorylation of rat myelin basic proteins, *J Neurosci Res 15*, 159-166.
21. Yates, A. J., Walters, J. D., Wood, C. L., and Johnson, J. D. (1989) Ganglioside modulation of cyclic AMP-dependent protein kinase and cyclic nucleotide phosphodiesterase in vitro, *J Neurochem 53*, 162-167.
22. Facci, L., Leon, A., Toffano, G., Sonnino, S., Ghidoni, R., and Tettamanti, G. (1984) Promotion of neuritogenesis in mouse neuroblastoma cells by exogenous gangliosides. Relationship between the effect and the cell association of ganglioside GM1, *J Neurochem 42*, 299-305.
23. Carter, H. E., Glick, F. J., Norris, W. P., and Phillips, G. E. (1947) Biochemisrty of sphingolipides. III. Structure of sphingosine, *J. Biol. Chem. 170*, 285-294.

24. Carter, H. E., Rothfus,J. A., Gigg, R. (1961) Biochemistry of the sphingolipids: XII. conversion of cerebrosides to ceramides and sphingosine; structure of Gaucher cerebroside, *J Lipid Res 2*, 228-234.
25. Kolesnick, R. N. (1991) Sphingomyelin and derivatives as cellular signals, *Prog Lipid Res 30*, 1-38.
26. Svennerholm, L. (1980) Structure and biology of cell membrane gangliosides, In *Cholera and related diarrheas 43rd Nobel Symposium*, pp 80-87, Stockholm.
27. Kolter, T., Proia, R. L., and Sandhoff, K. (2002) Combinatorial ganglioside biosynthesis, *J Biol Chem 277*, 25859-25862.
28. Hakomori, S. (1981) Glycosphingolipids in cellular interaction, differentiation, and oncogenesis, *Annu Rev Biochem 50*, 733-764.
29. van Meer, G., and Lisman, Q. (2002) Sphingolipid transport: rafts and translocators, *J Biol Chem 277*, 25855-25858.
30. Mukherjee, S., Kombrabail, M., Krishnamoorthy, G., and Chattopadhyay, A. (2007) Dynamics and heterogeneity of bovine hippocampal membranes: role of cholesterol and proteins, *Biochim Biophys Acta 1768*, 2130-2144.
31. Prinetti, A., Chigorno, V., Mauri, L., Loberto, N., and Sonnino, S. (2007) Modulation of cell functions by glycosphingolipid metabolic remodeling in the plasma membrane, *J Neurochem 103 Suppl 1*, 113-125.
32. van Meer, G., and Simons, K. (1988) Lipid polarity and sorting in epithelial cells, *J Cell Biochem 36*, 51-58.
33. van der Bijl, P., Lopes-Cardozo, M., and van Meer, G. (1996) Sorting of newly synthesized galactosphingolipids to the two surface domains of epithelial cells, *J Cell Biol 132*, 813-821.
34. Yates, A. J., and Rampersaud, A. (1998) Sphingolipids as receptor modulators. An overview, *Ann N Y Acad Sci 845*, 57-71.
35. Holmgren, J., Lonnroth, I., Mansson, J., and Svennerholm, L. (1975) Interaction of cholera toxin and membrane GM1 ganglioside of small intestine, *Proc Natl Acad Sci U S A 72*, 2520-2524.
36. Markwell, M. A., Svennerholm, L., and Paulson, J. C. (1981) Specific gangliosides function as host cell receptors for Sendai virus, *Proc Natl Acad Sci U S A 78*, 5406-5410.
37. Karlsson, K. A. (1989) Animal glycosphingolipids as membrane attachment sites for bacteria, *Annu Rev Biochem 58*, 309-350.
38. Ledeen, R. W., Wu, G., Lu, Z. H., Kozireski-Chuback, D., and Fang, Y. (1998) The role of GM1 and other gangliosides in neuronal differentiation. Overview and new finding, *Ann N Y Acad Sci 845*, 161-175.
39. Spiegel, S., Foster, D., and Kolesnick, R. (1996) Signal transduction through lipid second messengers, *Curr Opin Cell Biol 8*, 159-167.
40. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane, *J Biol Chem 273*, 5419-5422.
41. Sonnino, S., Prinetti, A., Mauri, L., Chigorno, V., and Tettamanti, G. (2006) Dynamic and structural properties of sphingolipids as driving forces for the formation of membrane domains, *Chem Rev 106*, 2111-2125.
42. Sonnino, S., Mauri, L., Chigorno, V., and Prinetti, A. (2007) Gangliosides as components of lipid membrane domains, *Glycobiology 17*, 1R-13R.
43. Ando, S. (1983) Gangliosides in the nervous system, *Neurochem Int 5*, 507-537.
44. Yu, R. K., Macala, L. J., Farooq, M., Sbaschnig-Agler, M., Norton, W. T., and Ledeen, R. W. (1989) Ganglioside and lipid composition of bulk-isolated rat and bovine oligodendroglia, *J Neurosci Res 23*, 136-141.
45. Ledeen, R. W., Golly, F., and Haley, J. E. (1992) Axon-myelin transfer of phospholipids and phospholipid precursors. Labeling of myelin phosphoinositides through axonal transport, *Mol Neurobiol 6*, 179-190.
46. Schwarz, A., and Futerman, A. H. (1996) The localization of gangliosides in neurons of the central nervous system: the use of anti-ganglioside antibodies, *Biochim Biophys Acta 1286*, 247-267.

47. Walkley, S. U., Siegel, D. A., Dobrenis, K., and Zervas, M. (1998) GM2 ganglioside as a regulator of pyramidal neuron dendritogenesis, *Ann N Y Acad Sci 845*, 188-199.
48. Walkley, S. U., Zervas, M., and Wiseman, S. (2000) Gangliosides as modulators of dendritogenesis in normal and storage disease-affected pyramidal neurons, *Cereb Cortex 10*, 1028-1037.
49. Norman, E., Cutler, R. G., Flannery, R., Wang, Y., and Mattson, M. P. (2010) Plasma membrane sphingomyelin hydrolysis increases hippocampal neuron excitability by sphingosine-1-phosphate mediated mechanisms, *J Neurochem 114*, 430-439.
50. Kojima, N., and Hakomori, S. (1991) Synergistic effect of two cell recognition systems: glycosphingolipid-glycosphingolipid interaction and integrin receptor interaction with pericellular matrix protein, *Glycobiology 1*, 623-630.
51. Dreyfus, H., Louis, J. C., Harth, S., and Mandel, P. (1980) Gangliosides in cultured neurons, *Neuroscience 5*, 1647-1655.
52. Ngamukote, S., Yanagisawa, M., Ariga, T., Ando, S., and Yu, R. K. (2007) Developmental changes of glycosphingolipids and expression of glycogenes in mouse brains, *J Neurochem 103*, 2327-2341.
53. Buccoliero, R., Bodennec, J., and Futerman, A. H. (2002) The role of sphingolipids in neuronal development: lessons from models of sphingolipid storage diseases, *Neurochem Res 27*, 565-574.
54. Yu, R. K., Bieberich, E., Xia, T., and Zeng, G. (2004) Regulation of ganglioside biosynthesis in the nervous system, *J Lipid Res 45*, 783-793.
55. Yavin, Z., and Yavin, E. (1978) Immunofluorescent patterns of dissociated rat embryo cerebral cells during development in surface culture: distinctive reactions with neurite and perikaryon cell membranes, *Dev Neurosci 1*, 31-40.
56. Prinetti, A., Chigorno, V., Prioni, S., Loberto, N., Marano, N., Tettamanti, G., and Sonnino, S. (2001) Changes in the lipid turnover, composition, and organization, as sphingolipid-enriched membrane domains, in rat cerebellar granule cells developing in vitro, *J Biol Chem 276*, 21136-21145.
57. Prinetti, A., Prioni, S., Chigorno, V., Karagogeos, D., Tettamanti, G., and Sonnino, S. (2001) Immunoseparation of sphingolipid-enriched membrane domains enriched in Src family protein tyrosine kinases and in the neuronal adhesion molecule TAG-1 by anti-GD3 ganglioside monoclonal antibody, *J Neurochem 78*, 1162-1167.
58. Prioni, S., Loberto, N., Prinetti, A., Chigorno, V., Guzzi, F., Maggi, R., Parenti, M., and Sonnino, S. (2002) Sphingolipid metabolism and caveolin expression in gonadotropin-releasing hormone-expressing GN11 and gonadotropin-releasing hormone-secreting GT1-7 neuronal cells, *Neurochem Res 27*, 831-840.
59. Riboni, L., Prinetti, A., Pitto, M., and Tettamanti, G. (1990) Patterns of endogenous gangliosides and metabolic processing of exogenous gangliosides in cerebellar granule cells during differentiation in culture, *Neurochem Res 15*, 1175-1183.
60. Rosenberg, A., Sauer, A., Noble, E. P., Gross, H. J., Chang, R., and Brossmer, R. (1992) Developmental patterns of ganglioside sialosylation coincident with neuritogenesis in cultured embryonic chick brain neurons, *J Biol Chem 267*, 10607-10612.
61. Pfeiffer, S. E., Warrington, A. E., and Bansal, R. (1993) The oligodendrocyte and its many cellular processes, *Trends Cell Biol 3*, 191-197.
62. Byrne, M. C., Ledeen, R. W., Roisen, F. J., Yorke, G., and Sclafani, J. R. (1983) Ganglioside-induced neuritogenesis: verification that gangliosides are the active agents, and comparison of molecular species, *J Neurochem 41*, 1214-1222.
63. Kadowaki, H., Evans, J. E., Rys-Sikora, K. E., and Koff, R. S. (1990) Effect of differentiation and cell density on glycosphingolipid class and molecular species composition of mouse neuroblastoma NB2a cells, *J Neurochem 54*, 2125-2137.
64. Tettamanti, G., and Riboni, L. (1994) Gangliosides turnover and neural cells function: a new perspective, *Prog Brain Res 101*, 77-100.
65. Tsuji, S., Yamashita, T., Tanaka, M., and Nagai, Y. (1988) Synthetic sialyl compounds as well as natural gangliosides induce neuritogenesis in a mouse neuroblastoma cell line (Neuro2a), *J Neurochem 50*, 414-423.

66. Ferrari, G., Fabris, M., and Gorio, A. (1983) Gangliosides enhance neurite outgrowth in PC12 cells, *Brain Res 284*, 215-221.
67. Mutoh, T., Hamano, T., Yano, S., Koga, H., Yamamoto, H., Furukawa, K., and Ledeen, R. W. (2002) Stable transfection of GM1 synthase gene into GM1-deficient NG108-15 cells, CR-72 cells, rescues the responsiveness of Trk-neurotrophin receptor to its ligand, NGF, *Neurochem Res 27*, 801-806.
68. Mutoh, T., Tokuda, A., Miyadai, T., Hamaguchi, M., and Fujiki, N. (1995) Ganglioside GM1 binds to the Trk protein and regulates receptor function, *Proc Natl Acad Sci U S A 92*, 5087-5091.
69. Wu, G. S., Lu, Z. H., and Ledeen, R. W. (1991) Correlation of gangliotetraose gangliosides with neurite forming potential of neuroblastoma cells, *Brain Res Dev Brain Res 61*, 217-228.
70. Wu, G., Lu, Z. H., and Ledeen, R. W. (1996) GM1 ganglioside modulates prostaglandin E1 stimulated adenylyl cyclase in neuro-2A cells, *Glycoconj J 13*, 235-239.
71. Prinetti, A., Iwabuchi, K., and Hakomori, S. (1999) Glycosphingolipid-enriched signaling domain in mouse neuroblastoma Neuro2a cells. Mechanism of ganglioside-dependent neuritogenesis, *J Biol Chem 274*, 20916-20924.
72. Lam, R. S., Shaw, A. R., and Duszyk, M. (2004) Membrane cholesterol content modulates activation of BK channels in colonic epithelia, *Biochim Biophys Acta 1667*, 241-248.
73. Naslavsky, N., Shmeeda, H., Friedlander, G., Yanai, A., Futerman, A. H., Barenholz, Y., and Taraboulos, A. (1999) Sphingolipid depletion increases formation of the scrapie prion protein in neuroblastoma cells infected with prions, *J Biol Chem 274*, 20763-20771.
74. Kasahara, K., Watanabe, K., Takeuchi, K., Kaneko, H., Oohira, A., Yamamoto, T., and Sanai, Y. (2000) Involvement of gangliosides in glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule TAG-1 signaling in lipid rafts, *J Biol Chem 275*, 34701-34709.
75. Inokuchi, J. I., Uemura, S., Kabayama, K., and Igarashi, Y. (2000) Glycosphingolipid deficiency affects functional microdomain formation in Lewis lung carcinoma cells, *Glycoconj J 17*, 239-245.
76. Mitsuzuka, K., Handa, K., Satoh, M., Arai, Y., and Hakomori, S. (2005) A specific microdomain ("glycosynapse 3") controls phenotypic conversion and reversion of bladder cancer cells through GM3-mediated interaction of alpha3beta1 integrin with CD9, *J Biol Chem 280*, 35545-35553.
77. Sato, T., Zakaria, A. M., Uemura, S., Ishii, A., Ohno-Iwashita, Y., Igarashi, Y., and Inokuchi, J. (2005) Role for up-regulated ganglioside biosynthesis and association of Src family kinases with microdomains in retinoic acid-induced differentiation of F9 embryonal carcinoma cells, *Glycobiology 15*, 687-699.
78. Toledo, M. S., Suzuki, E., Handa, K., and Hakomori, S. (2004) Cell growth regulation through GM3-enriched microdomain (glycosynapse) in human lung embryonal fibroblast WI38 and its oncogenic transformant VA13, *J Biol Chem 279*, 34655-34664.
79. Yanagisawa, M., Nakamura, K., and Taga, T. (2005) Glycosphingolipid synthesis inhibitor represses cytokine-induced activation of the Ras-MAPK pathway in embryonic neural precursor cells, *J Biochem 138*, 285-291.
80. Chang, M. C., Wisco, D., Ewers, H., Norden, C., and Winckler, B. (2006) Inhibition of sphingolipid synthesis affects kinetics but not fidelity of L1/NgCAM transport along direct but not transcytotic axonal pathways, *Mol Cell Neurosci 31*, 525-538.
81. Decker, L., Baron, W., and Ffrench-Constant, C. (2004) Lipid rafts: microenvironments for integrin-growth factor interactions in neural development, *Biochem Soc Trans 32*, 426-430.
82. Kilkus, J., Goswami, R., Testai, F. D., and Dawson, G. (2003) Ceramide in rafts (detergent-insoluble fraction) mediates cell death in neurotumor cell lines, *J Neurosci Res 72*, 65-75.
83. Ledesma, M. D., Simons, K., and Dotti, C. G. (1998) Neuronal polarity: essential role of protein-lipid complexes in axonal sorting, *Proc Natl Acad Sci U S A 95*, 3966-3971.
84. Inokuchi, J., Mizutani, A., Jimbo, M., Usuki, S., Yamagishi, K., Mochizuki, H., Muramoto, K., Kobayashi, K., Kuroda, Y., Iwasaki, K., Ohgami, Y., and Fujiwara, M. (1997) Up-regulation of ganglioside biosynthesis, functional synapse formation, and memory retention by a synthetic ceramide analog (L-PDMP), *Biochem Biophys Res Commun 237*, 595-600.
85. Rosner, D. L. (1998) Sleep disordered breathing: an overview, *J S C Med Assoc 94*, 542-545.

86. Mutoh, T., Rudkin, B. B., Koizumi, S., and Guroff, G. (1988) Nerve growth factor, a differentiating agent, and epidermal growth factor, a mitogen, increase the activities of different S6 kinases in PC12 cells, *J Biol Chem 263*, 15853-15856.
87. Jennemann, R., Sandhoff, R., Wang, S., Kiss, E., Gretz, N., Zuliani, C., Martin-Villalba, A., Jager, R., Schorle, H., Kenzelmann, M., Bonrouhi, M., Wiegandt, H., and Grone, H. J. (2005) Cell-specific deletion of glucosylceramide synthase in brain leads to severe neural defects after birth, *Proc Natl Acad Sci U S A 102*, 12459-12464.
88. Usuki, S., Hamanoue, M., Kohsaka, S., and Inokuchi, J. (1996) Induction of ganglioside biosynthesis and neurite outgrowth of primary cultured neurons by L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, *J Neurochem 67*, 1821-1830.
89. Kojima, N., Kurosawa, N., Nishi, T., Hanai, N., and Tsuji, S. (1994) Induction of cholinergic differentiation with neurite sprouting by de novo biosynthesis and expression of GD3 and b-series gangliosides in Neuro2a cells, *J Biol Chem 269*, 30451-30456.
90. Kanda, T., Ariga, T., Yamawaki, M., Pal, S., Katoh-Semba, R., and Yu, R. K. (1995) Effect of nerve growth factor and forskolin on glycosyltransferase activities and expression of a globo-series glycosphingolipid in PC12D pheochromocytoma cells, *J Neurochem 64*, 810-817.
91. Boldin, S. A., and Futerman, A. H. (2000) Up-regulation of glucosylceramide synthesis upon stimulation of axonal growth by basic fibroblast growth factor. Evidence for post-translational modification of glucosylceramide synthase, *J Biol Chem 275*, 9905-9909.
92. Proshin, S., Yamaguchi, K., Wada, T., and Miyagi, T. (2002) Modulation of neuritogenesis by ganglioside-specific sialidase (Neu 3) in human neuroblastoma NB-1 cells, *Neurochem Res 27*, 841-846.
93. Hasegawa, T., Yamaguchi, K., Wada, T., Takeda, A., Itoyama, Y., and Miyagi, T. (2000) Molecular cloning of mouse ganglioside sialidase and its increased expression in neuro2a cell differentiation, *J Biol Chem 275*, 14778.
94. Kopitz, J., Muhl, C., Ehemann, V., Lehmann, C., and Cantz, M. (1997) Effects of cell surface ganglioside sialidase inhibition on growth control and differentiation of human neuroblastoma cells, *Eur J Cell Biol 73*, 1-9.
95. Kopitz, J., Sinz, K., Brossmer, R., and Cantz, M. (1997) Partial characterization and enrichment of a membrane-bound sialidase specific for gangliosides from human brain tissue, *Eur J Biochem 248*, 527-534.
96. von Reitzenstein, C., Kopitz, J., Schuhmann, V., and Cantz, M. (2001) Differential functional relevance of a plasma membrane ganglioside sialidase in cholinergic and adrenergic neuroblastoma cell lines, *Eur J Biochem 268*, 326-333.
97. Da Silva, J. S., Hasegawa, T., Miyagi, T., Dotti, C. G., and Abad-Rodriguez, J. (2005) Asymmetric membrane ganglioside sialidase activity specifies axonal fate, *Nat Neurosci 8*, 606-615.
98. Rodriguez, J. A., Piddini, E., Hasegawa, T., Miyagi, T., and Dotti, C. G. (2001) Plasma membrane ganglioside sialidase regulates axonal growth and regeneration in hippocampal neurons in culture, *J Neurosci 21*, 8387-8395.
99. Monti, E., Bassi, M. T., Bresciani, R., Civini, S., Croci, G. L., Papini, N., Riboni, M., Zanchetti, G., Ballabio, A., Preti, A., Tettamanti, G., Venerando, B., and Borsani, G. (2004) Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family, *Genomics 83*, 445-453.
100. Seyrantepe, V., Landry, K., Trudel, S., Hassan, J. A., Morales, C. R., and Pshezhetsky, A. V. (2004) Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells, *J Biol Chem 279*, 37021-37029.
101. Yamaguchi, K., Hata, K., Koseki, K., Shiozaki, K., Akita, H., Wada, T., Moriya, S., and Miyagi, T. (2005) Evidence for mitochondrial localization of a novel human sialidase (NEU4), *Biochem J 390*, 85-93.
102. Geng, Y. Q., Guan, J. T., Xu, X. H., and Fu, Y. C. (2010) Senescence-associated beta-galactosidase activity expression in aging hippocampal neurons, *Biochem Biophys Res Commun 396*, 866-869.
103. Kusumi, A., and Suzuki, K. (2005) Toward understanding the dynamics of membrane-raft-based molecular interactions, *Biochim Biophys Acta 1746*, 234-251.

104. Rajendran, L., and Simons, K. (2005) Lipid rafts and membrane dynamics, *J Cell Sci 118*, 1099-1102.
105. Tsui-Pierchala, B. A., Encinas, M., Milbrandt, J., and Johnson, E. M., Jr. (2002) Lipid rafts in neuronal signaling and function, *Trends Neurosci 25*, 412-417.
106. Merrill, A. H., Jr. (2002) De novo sphingolipid biosynthesis: a necessary, but dangerous, pathway, *J Biol Chem 277*, 25843-25846.
107. Mandon, E. C., van Echten, G., Birk, R., Schmidt, R. R., and Sandhoff, K. (1991) Sphingolipid biosynthesis in cultured neurons. Down-regulation of serine palmitoyltransferase by sphingoid bases, *Eur J Biochem 198*, 667-674.
108. Nagiec, M. M., Baltisberger, J. A., Wells, G. B., Lester, R. L., and Dickson, R. C. (1994) The LCB2 gene of Saccharomyces and the related LCB1 gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis, *Proc Natl Acad Sci U S A 91*, 7899-7902.
109. Weiss, B., and Stoffel, W. (1997) Human and murine serine-palmitoyl-CoA transferase--cloning, expression and characterization of the key enzyme in sphingolipid synthesis, *Eur J Biochem 249*, 239-247.
110. Stoffel, W., LeKim, D., and Sticht, G. (1968) Metabolism of sphingosine bases. 8. Distribution, isolation and properties of D-3-oxosphinganine reductase. Stereospecificity of the NADPH-dependent reaction of 3-oxodihydrospingosine (2-amino-1-hydroxyoctadecane-3-one), *Hoppe Seylers Z Physiol Chem 349*, 1637-1644.
111. Rother, J., van Echten, G., Schwarzmann, G., and Sandhoff, K. (1992) Biosynthesis of sphingolipids: dihydroceramide and not sphinganine is desaturated by cultured cells, *Biochem Biophys Res Commun 189*, 14-20.
112. Merrill, A. H., Jr., and Wang, E. (1986) Biosynthesis of long-chain (sphingoid) bases from serine by LM cells. Evidence for introduction of the 4-trans-double bond after de novo biosynthesis of N-acylsphinganine(s), *J Biol Chem 261*, 3764-3769.
113. Shimeno, H., Soeda, S., Sakamoto, M., Kouchi, T., Kowakame, T., and Kihara, T. (1998) Partial purification and characterization of sphingosine N-acyltransferase (ceramide synthase) from bovine liver mitochondrion-rich fraction, *Lipids 33*, 601-605.
114. Geeraert, L., Mannaerts, G. P., and van Veldhoven, P. P. (1997) Conversion of dihydroceramide into ceramide: involvement of a desaturase, *Biochem J 327 (Pt 1)*, 125-132.
115. Michel, C., van Echten-Deckert, G., Rother, J., Sandhoff, K., Wang, E., and Merrill, A. H., Jr. (1997) Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-trans-double bond of sphingosine at the level of dihydroceramide, *J Biol Chem 272*, 22432-22437.
116. Mikami, T., Kashiwagi, M., Tsuchihashi, K., Akino, T., and Gasa, S. (1998) Substrate specificity and some other enzymatic properties of dihydroceramide desaturase (ceramide synthase) in fetal rat skin, *J Biochem (Tokyo) 123*, 906-911.
117. Merrill, A. H., Jr. (1983) Characterization of serine palmitoyltransferase activity in Chinese hamster ovary cells, *Biochim Biophys Acta 754*, 284-291.
118. Merrill, A. H., Jr., and Jones, D. D. (1990) An update of the enzymology and regulation of sphingomyelin metabolism, *Biochim Biophys Acta 1044*, 1-12.
119. Spence, M. W. (1993) Sphingomyelinases, *Adv Lipid Res 26*, 3-23.
120. Jeckel, D., Karrenbauer, A., Burger, K. N., van Meer, G., and Wieland, F. (1992) Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions, *J Cell Biol 117*, 259-267.
121. Warnock, D. E., Lutz, M. S., Blackburn, W. A., Young, W. W., Jr., and Baenziger, J. U. (1994) Transport of newly synthesized glucosylceramide to the plasma membrane by a non-Golgi pathway, *Proc Natl Acad Sci U S A 91*, 2708-2712.
122. Nomura, T., Takizawa, M., Aoki, J., Arai, H., Inoue, K., Wakisaka, E., Yoshizuka, N., Imokawa, G., Dohmae, N., Takio, K., Hattori, M., and Matsuo, N. (1998) Purification, cDNA cloning, and expression of UDP-Gal: glucosylceramide beta-1,4-galactosyltransferase from rat brain, *J Biol Chem 273*, 13570-13577.
123. Lannert, H., Gorgas, K., Meissner, I., Wieland, F. T., and Jeckel, D. (1998) Functional organization of the Golgi apparatus in glycosphingolipid biosynthesis. Lactosylceramide and subsequent glycosphingolipids are formed in the lumen of the late Golgi, *J Biol Chem 273*, 2939-2946.

124. Hakomori, S. (1990) Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions, *J Biol Chem 265*, 18713-18716.
125. Yu, R. K., Yanagisawa, M., and Ariga, T. (2007) Glycosphingolipid Structures, In *Comprehensive Glycoscience* (Johannis, P. K., Ed.), pp 73-122, Elsevier, Oxford.
126. Kaufman, B., Basu, S., and Roseman, S. (1968) Enzymatic synthesis of disialogangliosides from monosialogangliosides by sialyltransferases from embryonic chicken brain, *J Biol Chem 243*, 5804-5807.
127. Yip, M. C., and Dain, J. A. (1969) The enzymic synthesis of ganglioside. 1. Brain uridine diphosphate D-galactose: N-acetyl-galactosaminyl-galactosyl-glucosyl-ceramide galactosyl transferase, *Lipids 4*, 270-277.
128. Gillard, B. K., Clement, R. G., and Marcus, D. M. (1998) Variations among cell lines in the synthesis of sphingolipids in de novo and recycling pathways, *Glycobiology 8*, 885-890.
129. Lloyd, K. O., and Furukawa, K. (1998) Biosynthesis and functions of gangliosides: recent advances, *Glycoconj J 15*, 627-636.
130. Sheikh, K. A., Sun, J., Liu, Y., Kawai, H., Crawford, T. O., Proia, R. L., Griffin, J. W., and Schnaar, R. L. (1999) Mice lacking complex gangliosides develop Wallerian degeneration and myelination defects, *Proc Natl Acad Sci U S A 96*, 7532-7537.
131. Yamashita, T., Wada, R., Sasaki, T., Deng, C., Bierfreund, U., Sandhoff, K., and Proia, R. L. (1999) A vital role for glycosphingolipid synthesis during development and differentiation, *Proc Natl Acad Sci U S A 96*, 9142-9147.
132. Huwiler, A., Kolter, T., Pfeilschifter, J., and Sandhoff, K. (2000) Physiology and pathophysiology of sphingolipid metabolism and signaling, *Biochim Biophys Acta 1485*, 63-99.
133. Peters, C., and von Figura, K. (1994) Biogenesis of lysosomal membranes, *FEBS Lett 346*, 108-114.
134. Carlsson, S. R., Roth, J., Piller, F., and Fukuda, M. (1988) Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylactosaminoglycan, *J Biol Chem 263*, 18911-18919.
135. Henning, R., and Stoffel, W. (1973) Glycosphingolipids in lysosomal membranes, *Hoppe Seylers Z Physiol Chem 354*, 760-770.
136. Furst, W., and Sandhoff, K. (1992) Activator proteins and topology of lysosomal sphingolipid catabolism, *Biochim Biophys Acta 1126*, 1-16.
137. Burkhardt, J. K., Huttler, S., Klein, A., Mobius, W., Habermann, A., Griffiths, G., and Sandhoff, K. (1997) Accumulation of sphingolipids in SAP-precursor (prosaposin)-deficient fibroblasts occurs as intralysosomal membrane structures and can be completely reversed by treatment with human SAP-precursor, *Eur J Cell Biol 73*, 10-18.
138. Mobius, W., Herzog, V., Sandhoff, K., and Schwarzmann, G. (1999) Intracellular distribution of a biotin-labeled ganglioside, GM1, by immunoelectron microscopy after endocytosis in fibroblasts, *J Histochem Cytochem 47*, 1005-1014.
139. Wilkening, G., Linke, T., Uhlhorn-Dierks, G., and Sandhoff, K. (2000) Degradation of membrane-bound ganglioside GM1. Stimulation by bis(monoacylglycero)phosphate and the activator proteins SAP-B and GM2-AP, *J Biol Chem 275*, 35814-35819.
140. Wada, T., Yoshikawa, Y., Tokuyama, S., Kuwabara, M., Akita, H., and Miyagi, T. (1999) Cloning, expression, and chromosomal mapping of a human ganglioside sialidase, *Biochem Biophys Res Commun 261*, 21-27.
141. Miyagi, T., Wada, T., Iwamatsu, A., Hata, K., Yoshikawa, Y., Tokuyama, S., and Sawada, M. (1999) Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides, *J Biol Chem 274*, 5004-5011.
142. Kytzia, H. J., and Sandhoff, K. (1985) Evidence for two different active sites on human beta-hexosaminidase A. Interaction of GM2 activator protein with beta-hexosaminidase A, *J Biol Chem 260*, 7568-7572.
143. Proia, R. L., and Soravia, E. (1987) Organization of the gene encoding the human beta-hexosaminidase alpha-chain, *J Biol Chem 262*, 5677-5681.

144. Proia, R. L. (1988) Gene encoding the human beta-hexosaminidase beta chain: extensive homology of intron placement in the alpha- and beta-chain genes, *Proc Natl Acad Sci U S A 85*, 1883-1887.
145. Bairoch, A. (1999) The ENZYME data bank in 1999, *Nucleic Acids Res 27*, 310-311.
146. Hepbildikler, S. T., Sandhoff, R., Kolzer, M., Proia, R. L., and Sandhoff, K. (2002) Physiological substrates for human lysosomal beta -hexosaminidase S, *J Biol Chem 277*, 2562-2572.
147. Kolodny, E. H., Kanfer, J., Quirk, J. M., and Brady, R. O. (1971) Properties of a particle-bound enzyme from rat intestine that cleaves sialic acid from Tay-Sachs ganglioside, *J Biol Chem 246*, 1426-1431.
148. Riboni, L., Caminiti, A., Bassi, R., and Tettamanti, G. (1995) The degradative pathway of gangliosides GM1 and GM2 in Neuro2a cells by sialidase, *J Neurochem 64*, 451-454.
149. Sango, K., Yamanaka, S., Hoffmann, A., Okuda, Y., Grinberg, A., Westphal, H., McDonald, M. P., Crawley, J. N., Sandhoff, K., Suzuki, K., and Proia, R. L. (1995) Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism, *Nat Genet 11*, 170-176.
150. Miyagi, T., and Tsuiki, S. (1986) Evidence for sialidase hydrolyzing gangliosides GM2 and GM1 in rat liver plasma membrane, *FEBS Lett 206*, 223-228.
151. Fingerhut, R., van der Horst, G. T., Verheijen, F. W., and Conzelmann, E. (1992) Degradation of gangliosides by the lysosomal sialidase requires an activator protein, *Eur J Biochem 208*, 623-629.
152. Zschoche, A., Furst, W., Schwarzmann, G., and Sanhoff, K. (1994) Hydrolysis of lactosylceramide by human galactosylceramidase and GM1-beta-galactosidase in a detergent-free system and its stimulation by sphingolipid activator proteins, sap-B and sap-C. Activator proteins stimulate lactosylceramide hydrolysis, *Eur J Biochem 222*, 83-90.
153. Linke, T., Wilkening, G., Sadeghlar, F., Mozcall, H., Bernardo, K., Schuchman, E., and Sandhoff, K. (2001) Interfacial regulation of acid ceramidase activity. Stimulation of ceramide degradation by lysosomal lipids and sphingolipid activator proteins, *J Biol Chem 276*, 5760-5768.
154. Ito, M., and Yamagata, T. (1986) A novel glycosphingolipid-degrading enzyme cleaves the linkage between the oligosaccharide and ceramide of neutral and acidic glycosphingolipids, *J Biol Chem 261*, 14278-14282.
155. Zhou, B., Li, S. C., Laine, R. A., Huang, R. T., and Li, Y. T. (1989) Isolation and characterization of ceramide glycanase from the leech, Macrobdella decora, *J Biol Chem 264*, 12272-12277.
156. Ito, M., Ikegami, Y., and Yamagata, T. (1991) Activator proteins for glycosphingolipid hydrolysis by endoglycoceramidases. Elucidation of biological functions of cell-surface glycosphingolipids in situ by endoglycoceramidases made possible using these activator proteins, *J Biol Chem 266*, 7919-7926.
157. Basu, M., Girzadas, M., Dastgheib, S., Baker, J., Rossi, F., Radin, N. S., and Basu, S. (1997) Ceramide glycanase from rat mammary tissues: inhibition by PPMP(D-/L-) and its probable role in signal transduction, *Indian J Biochem Biophys 34*, 142-149.
158. Basu, M., Kelly, P., Girzadas, M., Li, Z., and Basu, S. (2000) Properties of animal ceramide glycanases, *Methods Enzymol 311*, 287-297.
159. Dastgheib, S., Basu, S. S., Li, Z., Basu, M., and Basu, S. (2000) Analyses of glycosphingolipids using clam, Mercenaria mercenaria, ceramide glycanase, *Methods Enzymol 312*, 196-205.
160. Hirabayashi, Y., Kimura, M., Matsumoto, M., Yamamoto, K., Kadowaki, S., and Tochikura, T. (1988) A novel glycosphingolipid hydrolyzing enzyme, glycosphingolipid ceramide deacylase, which cleaves the linkage between the fatty acid and sphingosine base in glycosphingolipids, *J Biochem 103*, 1-4.
161. Furusato, M., Sueyoshi, N., Mitsutake, S., Sakaguchi, K., Kita, K., Okino, N., Ichinose, S., Omori, A., and Ito, M. (2002) Molecular cloning and characterization of sphingolipid ceramide N-deacylase from a marine bacterium, Shewanella alga G8, *J Biol Chem 277*, 17300-17307.
162. Rodriguez-Lafrasse, C., and Vanier, M. T. (1999) Sphingosylphosphorylcholine in Niemann-Pick disease brain: accumulation in type A but not in type B, *Neurochem Res 24*, 199-205.
163. Lombardo, A., Caimi, L., Marchesini, S., Goi, G. C., and Tettamanti, G. (1980) Enzymes of lysosomal origin in human plasma and serum: assay conditions and parameters influencing the assay, *Clin Chim Acta 108*, 337-346.

164. Weng, S., and Spiro, R. G. (1996) Endoplasmic reticulum kifunensine-resistant alpha-mannosidase is enzymatically and immunologically related to the cytosolic alpha-mannosidase, *Arch Biochem Biophys 325*, 113-123.
165. Gopalan, V., Glew, R. H., Libell, D. P., and DePetro, J. J. (1989) The dual effects of alcohols on the kinetic properties of guinea pig liver cytosolic beta-glucosidase, *J Biol Chem 264*, 15418-15422.
166. Willemsen, R., Brunken, R., Sorber, C. W., Hoogeveen, A. T., Wisselaar, H. A., Van Dongen, J. M., and Reuser, A. J. (1991) A quantitative immunoelectronmicroscopic study on soluble, membrane-associated and membrane-bound lysosomal enzymes in human intestinal epithelial cells, *Histochem J 23*, 467-473.
167. Venerando, B., Goi, G. C., Preti, A., Fiorilli, A., Lombardo, A., and Tettamanti, G. (1982) Cytosolic sialidase in developing rat forebrain, *Neurochem Int 4*, 313-320.
168. Venerando, B., Fiorilli, A., Croci, G. L., and Tettamanti, G. (1997) Presence in human erythrocyte membranes of a novel form of sialidase acting optimally at neutral pH, *Blood 90*, 2047-2056.
169. Mahuran, D., Novak, A., and Lowden, J. A. (1985) The lysosomal hexosaminidase isozymes, *Isozymes Curr Top Biol Med Res 12*, 229-288.
170. Chiarini, A., Fiorilli, A., Di Francesco, L., Venerando, B., and Tettamanti, G. (1993) Human erythrocyte sialidase is linked to the plasma membrane by a glycosylphosphatidylinositol anchor and partly located on the outer surface, *Glycoconj J 10*, 64-71.
171. Sinnott, M. J. (1990) An aid to the management of pediatric trauma in peripheral centers--a proposal for a pediatric trauma board, *J Emerg Med 8*, 413-417.
172. Henrissat, B., Callebaut, I., Fabrega, S., Lehn, P., Mornon, J. P., and Davies, G. (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases, *Proc Natl Acad Sci U S A 92*, 7090-7094.
173. Davies, G., and Henrissat, B. (1995) Structures and mechanisms of glycosyl hydrolases, *Structure 3*, 853-859.
174. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave, *Nat Rev Mol Cell Biol 9*, 112-124.
175. Levade, T., and Jaffrezou, J. P. (1999) Signalling sphingomyelinases: which, where, how and why?, *Biochim Biophys Acta 1438*, 1-17.
176. Goni, F. M., and Alonso, A. (2002) Sphingomyelinases: enzymology and membrane activity, *FEBS Lett 531*, 38-46.
177. Huitema, K., van den Dikkenberg, J., Brouwers, J. F., and Holthuis, J. C. (2004) Identification of a family of animal sphingomyelin synthases, *Embo J 23*, 33-44.
178. Kalka, D., von Reitzenstein, C., Kopitz, J., and Cantz, M. (2001) The plasma membrane ganglioside sialidase cofractionates with markers of lipid rafts, *Biochem Biophys Res Commun 283*, 989-993.
179. Papini, N., Anastasia, L., Tringali, C., Croci, G., Bresciani, R., Yamaguchi, K., Miyagi, T., Preti, A., Prinetti, A., Prioni, S., Sonnino, S., Tettamanti, G., Venerando, B., and Monti, E. (2004) The plasma membrane-associated sialidase MmNEU3 modifies the ganglioside pattern of adjacent cells supporting its involvement in cell-to-cell interactions, *J Biol Chem 279*, 16989-16995.
180. Preti, A., Fiorilli, A., Lombardo, A., Caimi, L., and Tettamanti, G. (1980) Occurrence of sialyltransferase activity in the synaptosomal membranes prepared from calf brain cortex, *J Neurochem 35*, 281-296.
181. Matsui, Y., Lombard, D., Massarelli, R., Mandel, P., and Dreyfus, H. (1986) Surface glycosyltransferase activities during development of neuronal cell cultures, *J Neurochem 46*, 144-150.
182. Durrie, R., Saito, M., and Rosenberg, A. (1988) Endogenous glycosphingolipid acceptor specificity of sialosyltransferase systems in intact Golgi membranes, synaptosomes, and synaptic plasma membranes from rat brain, *Biochemistry 27*, 3759-3764.
183. Durrie, R., and Rosenberg, A. (1989) Anabolic sialosylation of gangliosides in situ in rat brain cortical slices, *J Lipid Res 30*, 1259-1266.

184. Iwamori, M., and Iwamori, Y. (2005) Changes in the glycolipid composition and characteristic activation of GM3 synthase in the thymus of mouse after administration of dexamethasone, *Glycoconj J 22*, 119-126.
185. Mencarelli, S., Cavalieri, C., Magini, A., Tancini, B., Basso, L., Lemansky, P., Hasilik, A., Li, Y. T., Chigorno, V., Orlacchio, A., Emiliani, C., and Sonnino, S. (2005) Identification of plasma membrane associated mature beta-hexosaminidase A, active towards GM2 ganglioside, in human fibroblasts, *FEBS Lett 579*, 5501-5506.
186. Kong, Y., Li, R., and Ladisch, S. (1998) Natural forms of shed tumor gangliosides, *Biochim Biophys Acta 1394*, 43-56.
187. Deng, W., Li, R., and Ladisch, S. (2000) Influence of cellular ganglioside depletion on tumor formation, *J Natl Cancer Inst 92*, 912-917.
188. Chigorno, V., Giannotta, C., Ottico, E., Sciannamblo, M., Mikulak, J., Prinetti, A., and Sonnino, S. (2005) Sphingolipid uptake by cultured cells: complex aggregates of cell sphingolipids with serum proteins and lipoproteins are rapidly catabolized, *J Biol Chem 280*, 2668-2675.
189. Hakomori, S. (1996) Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism, *Cancer Res 56*, 5309-5318.
190. Hakomori, S. (2002) Glycosylation defining cancer malignancy: new wine in an old bottle, *Proc Natl Acad Sci U S A 99*, 10231-10233.
191. Lukong, K. E., Seyrantepe, V., Landry, K., Trudel, S., Ahmad, A., Gahl, W. A., Lefrancois, S., Morales, C. R., and Pshezhetsky, A. V. (2001) Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail, *J Biol Chem 276*, 46172-46181.
192. Cordero, O. J., Merino, A., Paez de la Cadena, M., Bugia, B., Nogueira, M., Vinuela, J. E., Martinez-Zorzano, V. S., de Carlos, A., and Rodriguez-Berrocal, F. J. (2001) Cell surface human alpha-L-fucosidase, *Eur J Biochem 268*, 3321-3331.
193. Monti, E., Preti, A., Venerando, B., and Borsani, G. (2002) Recent development in mammalian sialidase molecular biology, *Neurochem Res 27*, 649-663.
194. Miyagi, T., Wada, T., Yamaguchi, K., Shiozaki, K., Sato, I., Kakugawa, Y., Yamanami, H., and Fujiya, T. (2008) Human sialidase as a cancer marker, *Proteomics 8*, 3303-3311.
195. Miyagi, T., Wada, T., and Yamaguchi, K. (2008) Roles of plasma membrane-associated sialidase NEU3 in human cancers, *Biochim Biophys Acta 1780*, 532-537.
196. Ueno, S., Saito, S., Wada, T., Yamaguchi, K., Satoh, M., Arai, Y., and Miyagi, T. (2006) Plasma membrane-associated sialidase is up-regulated in renal cell carcinoma and promotes interleukin-6-induced apoptosis suppression and cell motility, *J Biol Chem 281*, 7756-7764.
197. Kakugawa, Y., Wada, T., Yamaguchi, K., Yamanami, H., Ouchi, K., Sato, I., and Miyagi, T. (2002) Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression, *Proc Natl Acad Sci U S A 99*, 10718-10723.
198. Venerando, B., Fiorilli, A., Croci, G., Tringali, C., Goi, G., Mazzanti, L., Curatola, G., Segalini, G., Massaccesi, L., Lombardo, A., and Tettamanti, G. (2002) Acidic and neutral sialidase in the erythrocyte membrane of type 2 diabetic patients, *Blood 99*, 1064-1070.
199. Tringali, C., Lupo, B., Anastasia, L., Papini, N., Monti, E., Bresciani, R., Tettamanti, G., and Venerando, B. (2007) Expression of sialidase Neu2 in leukemic K562 cells induces apoptosis by impairing Bcr-Abl/Src kinases signaling, *J Biol Chem 282*, 14364-14372.
200. Tringali, C., Anastasia, L., Papini, N., Bianchi, A., Ronzoni, L., Cappellini, M. D., Monti, E., Tettamanti, G., and Venerando, B. (2007) Modification of sialidase levels and sialoglycoconjugate pattern during erythroid and erytroleukemic cell differentiation, *Glycoconj J 24*, 67-79.
201. Chigorno, V., Cardace, G., Pitto, M., Sonnino, S., Ghidoni, R., and Tettamanti, G. (1986) A radiometric assay for ganglioside sialidase applied to the determination of the enzyme subcellular location in cultured human fibroblasts, *Anal Biochem 153*, 283-294.
202. Schengrund, C. L., and Repman, M. A. (1982) Density-dependent changes in gangliosides and sialidase activity of murine neuroblastoma cells, *J Neurochem 39*, 940-947.

203. Valaperta, R., Valsecchi, M., Rocchetta, F., Aureli, M., Prioni, S., Prinetti, A., Chigorno, V., and Sonnino, S. (2007) Induction of axonal differentiation by silencing plasma membrane-associated sialidase Neu3 in neuroblastoma cells, *J Neurochem 100*, 708-719.
204. Aureli, M., Loberto, N., P., L., Chigorno, V., Prinetti, A., and Sonnino, S. (2010) Cell surface sphingolipid glycohydrolases in neuronal differentation and aging in culture, *J Neurochem*.
205. Coates, P. J. (2002) Markers of senescence?, *J Pathol 196*, 371-373.
206. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo, *Proc Natl Acad Sci U S A 92*, 9363-9367.
207. Severino, J., Allen, R. G., Balin, S., Balin, A., and Cristofalo, V. J. (2000) Is beta-galactosidase staining a marker of senescence in vitro and in vivo?, *Exp Cell Res 257*, 162-171.
208. Geng, Y. Q., Guan, J. T., Xu, X. H., and Fu, Y. C. Senescence-associated beta-galactosidase activity expression in aging hippocampal neurons, *Biochem Biophys Res Commun 396*, 866-869.
209. Neufeld, E. F. (1991) Lysosomal storage diseases, *Annu Rev Biochem 60*, 257-280.
210. Daniels, L. B., Coyle, P. J., Chiao, Y. B., Glew, R. H., and Labow, R. S. (1981) Purification and characterization of a cytosolic broad specificity beta-glucosidase from human liver, *J Biol Chem 256*, 13004-13013.
211. Boot, R. G., Verhoek, M., Donker-Koopman, W., Strijland, A., van Marle, J., Overkleeft, H. S., Wennekes, T., and Aerts, J. M. (2007) Identification of the non-lysosomal glucosylceramidase as beta-glucosidase 2, *J Biol Chem 282*, 1305-1312.
212. Overkleeft, H. S., Renkema, G. H., Neele, J., Vianello, P., Hung, I. O., Strijland, A., van der Burg, A. M., Koomen, G. J., Pandit, U. K., and Aerts, J. M. (1998) Generation of specific deoxynojirimycin-type inhibitors of the non-lysosomal glucosylceramidase, *J Biol Chem 273*, 26522-26527.
213. Clarke, C. J., Truong, T. G., and Hannun, Y. A. (2007) Role for neutral sphingomyelinase-2 in tumor necrosis factor alpha-stimulated expression of vascular cell adhesion molecule-1 (VCAM) and intercellular adhesion molecule-1 (ICAM) in lung epithelial cells: p38 MAPK is an upstream regulator of nSMase2, *J Biol Chem 282*, 1384-1396.
214. Corcoran, C. A., He, Q., Ponnusamy, S., Ogretmen, B., Huang, Y., and Sheikh, M. S. (2008) Neutral sphingomyelinase-3 is a DNA damage and nongenotoxic stress-regulated gene that is deregulated in human malignancies, *Mol Cancer Res 6*, 795-807.
215. Krut, O., Wiegmann, K., Kashkar, H., Yazdanpanah, B., and Kronke, M. (2006) Novel tumor necrosis factor-responsive mammalian neutral sphingomyelinase-3 is a C-tail-anchored protein, *J Biol Chem 281*, 13784-13793.
216. Stoffel, W., Jenke, B., Block, B., Zumbansen, M., and Koebke, J. (2005) Neutral sphingomyelinase 2 (smpd3) in the control of postnatal growth and development, *Proc Natl Acad Sci U S A 102*, 4554-4559.
217. Kim, W. J., Okimoto, R. A., Purton, L. E., Goodwin, M., Haserlat, S. M., Dayyani, F., Sweetser, D. A., McClatchey, A. I., Bernard, O. A., Look, A. T., Bell, D. W., Scadden, D. T., and Haber, D. A. (2008) Mutations in the neutral sphingomyelinase gene SMPD3 implicate the ceramide pathway in human leukemias, *Blood 111*, 4716-4722.
218. Karakashian, A. A., Giltiay, N. V., Smith, G. M., and Nikolova-Karakashian, M. N. (2004) Expression of neutral sphingomyelinase-2 (NSMase-2) in primary rat hepatocytes modulates IL-beta-induced JNK activation, *FASEB J 18*, 968-970.
219. Marchesini, N., and Hannun, Y. A. (2004) Acid and neutral sphingomyelinases: roles and mechanisms of regulation, *Biochem Cell Biol 82*, 27-44.
220. Tani, M., and Hannun, Y. A. (2007) Neutral sphingomyelinase 2 is palmitoylated on multiple cysteine residues. Role of palmitoylation in subcellular localization, *J Biol Chem 282*, 10047-10056.
221. Reddy, A., Caler, E. V., and Andrews, N. W. (2001) Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes, *Cell 106*, 157-169.
222. Cardone, R. A., Casavola, V., and Reshkin, S. J. (2005) The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis, *Nat Rev Cancer 5*, 786-795.

223. Chiche, J., Ilc, K., Laferriere, J., Trottier, E., Dayan, F., Mazure, N. M., Brahimi-Horn, M. C., and Pouyssegur, J. (2009) Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH, *Cancer Res 69*, 358-368.
224. Casey, J. R., Grinstein, S., and Orlowski, J. (2010) Sensors and regulators of intracellular pH, *Nat Rev Mol Cell Biol 11*, 50-61.
225. Harguindey, S., Arranz, J. L., Wahl, M. L., Orive, G., and Reshkin, S. J. (2009) Proton transport inhibitors as potentially selective anticancer drugs, *Anticancer Res 29*, 2127-2136.
226. Chiche, J., Brahimi-Horn, M. C., and Pouyssegur, J. (2010) Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer, *J Cell Mol Med 14*, 771-794.
227. Mauri, L., Casellato, R., Kirschner, G., and Sonnino, S. (1999) A procedure for the preparation of GM3 ganglioside from GM1-lactone, *Glycoconj J 16*, 197-203.
228. Prinetti, A., Basso, L., Appierto, V., Villani, M. G., Valsecchi, M., Loberto, N., Prioni, S., Chigorno, V., Cavadini, E., Formelli, F., and Sonnino, S. (2003) Altered sphingolipid metabolism in N-(4-hydroxyphenyl)-retinamide-resistant A2780 human ovarian carcinoma cells, *J Biol Chem 278*, 5574-5583.
229. Leroy, J. G., Ho, M. W., MacBrinn, M. C., Zielke, K., Jacob, J., and O'Brien, J. S. (1972) I-cell disease: biochemical studies, *Pediatr Res 6*, 752-757.
230. Gallo, V., Ciotti, M. T., Coletti, A., Aloisi, F., and Levi, G. (1982) Selective release of glutamate from cerebellar granule cells differentiating in culture, *Proc Natl Acad Sci U S A 79*, 7919-7923.
231. Boyle, P. J., Ma, R., Tuteja, N., Banerjee, S., and Basu, S. (2006) Apoptosis of human breast carcinoma cells in the presence of cis-platin and L-/D-PPMP: IV. Modulation of replication complexes and glycolipid: Glycosyltransferases, *Glycoconj J 23*, 175-187.
232. Prinetti, A., Chigorno, V., Tettamanti, G., and Sonnino, S. (2000) Sphingolipid-enriched membrane domains from rat cerebellar granule cells differentiated in culture. A compositional study, *J Biol Chem 275*, 11658-11665.
233. Prinetti, A., Marano, N., Prioni, S., Chigorno, V., Mauri, L., Casellato, R., Tettamanti, G., and Sonnino, S. (2000) Association of Src-family protein tyrosine kinases with sphingolipids in rat cerebellar granule cells differentiated in culture, *Glycoconj J 17*, 223-232.
234. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J Biol Chem 193*, 265-275.
235. Sonnino, S., Aureli, M., Loberto, N., Chigorno, V., and Prinetti, A. (2010) Fine tuning of cell functions through remodeling of glycosphingolipids by plasma membrane-associated glycohydrolases, *FEBS Lett 584*, 1914-1922.
236. Glunde, K., Guggino, S. E., Solaiyappan, M., Pathak, A. P., Ichikawa, Y., and Bhujwalla, Z. M. (2003) Extracellular acidification alters lysosomal trafficking in human breast cancer cells, *Neoplasia 5*, 533-545.
237. Muller, R., Heinrich, M., Heck, S., Blohm, D., and Richter-Landsberg, C. (1997) Expression of microtubule-associated proteins MAP2 and tau in cultured rat brain oligodendrocytes, *Cell Tissue Res 288*, 239-249.
238. Riederer, B., and Matus, A. (1985) Differential expression of distinct microtubule-associated proteins during brain development, *Proc Natl Acad Sci U S A 82*, 6006-6009.
239. Kosik, K. S., and Finch, E. A. (1987) MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites: an immunocytochemical study of cultured rat cerebrum, *J Neurosci 7*, 3142-3153.
240. Georgieff, I. S., Liem, R. K., Couchie, D., Mavilia, C., Nunez, J., and Shelanski, M. L. (1993) Expression of high molecular weight tau in the central and peripheral nervous systems, *J Cell Sci 105 (Pt 3)*, 729-737.
241. Furley, A. J., Morton, S. B., Manalo, D., Karagogeos, D., Dodd, J., and Jessell, T. M. (1990) The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity, *Cell 61*, 157-170.

242. Karagogeos, D., Morton, S. B., Casano, F., Dodd, J., and Jessell, T. M. (1991) Developmental expression of the axonal glycoprotein TAG-1: differential regulation by central and peripheral neurons in vitro, *Development 112*, 51-67.
243. Loers, G., Chen, S., Grumet, M., and Schachner, M. (2005) Signal transduction pathways implicated in neural recognition molecule L1 triggered neuroprotection and neuritogenesis, *J Neurochem 92*, 1463-1476.
244. Lieberoth, A., Splittstoesser, F., Katagihallimath, N., Jakovcevski, I., Loers, G., Ranscht, B., Karagogeos, D., Schachner, M., and Kleene, R. (2009) Lewis(x) and alpha2,3-sialyl glycans and their receptors TAG-1, Contactin, and L1 mediate CD24-dependent neurite outgrowth, *J Neurosci 29*, 6677-6690.
245. Malhotra, J. D., Tsiotra, P., Karagogeos, D., and Hortsch, M. (1998) Cis-activation of L1-mediated ankyrin recruitment by TAG-1 homophilic cell adhesion, *J Biol Chem 273*, 33354-33359.
246. Craven, S. E., El-Husseini, A. E., and Bredt, D. S. (1999) Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs, *Neuron 22*, 497-509.
247. Charych, E. I., Akum, B. F., Goldberg, J. S., Jornsten, R. J., Rongo, C., Zheng, J. Q., and Firestein, B. L. (2006) Activity-independent regulation of dendrite patterning by postsynaptic density protein PSD-95, *J Neurosci 26*, 10164-10176.
248. Yasuda, Y., Tokita, Y., Aono, S., Matsui, F., Ono, T., Sonta, S., Watanabe, E., Nakanishi, Y., and Oohira, A. (1998) Cloning and chromosomal mapping of the human gene of neuroglycan C (NGC), a neural transmembrane chondroitin sulfate proteoglycan with an EGF module, *Neurosci Res 32*, 313-322.
249. Yamauchi, S., Tokita, Y., Aono, S., Matsui, F., Shuo, T., Ito, H., Kato, K., Kasahara, K., and Oohira, A. (2002) Phosphorylation of neuroglycan C, a brain-specific transmembrane chondroitin sulfate proteoglycan, and its localization in the lipid rafts, *J Biol Chem 277*, 20583-20590.
250. Ito, S., and Takeichi, M. (2009) Dendrites of cerebellar granule cells correctly recognize their target axons for synaptogenesis in vitro, *Proc Natl Acad Sci U S A 106*, 12782-12787.
251. Yu, R. K. (1994) Development regulation of ganglioside metabolism, *Prog Brain Res 101*, 31-44.
252. Yu, R. K., Nakatani, Y., and Yanagisawa, M. (2009) The role of glycosphingolipid metabolism in the developing brain, *J Lipid Res 50 Suppl*, S440-445.
253. Schwarz, A., Rapaport, E., Hirschberg, K., and Futerman, A. H. (1995) A regulatory role for sphingolipids in neuronal growth. Inhibition of sphingolipid synthesis and degradation have opposite effects on axonal branching, *J Biol Chem 270*, 10990-10998.
254. Hakomori, S., and Igarashi, Y. (1995) Functional role of glycosphingolipids in cell recognition and signaling, *J Biochem 118*, 1091-1103.
255. Milhas, D., Clarke, C. J., and Hannun, Y. A. (2010) Sphingomyelin metabolism at the plasma membrane: implications for bioactive sphingolipids, *FEBS Lett 584*, 1887-1894.
256. van Weely, S., Brandsma, M., Strijland, A., Tager, J. M., and Aerts, J. M. (1993) Demonstration of the existence of a second, non-lysosomal glucocerebrosidase that is not deficient in Gaucher disease, *Biochim Biophys Acta 1181*, 55-62.
257. Idone, V., Tam, C., and Andrews, N. W. (2008) Two-way traffic on the road to plasma membrane repair, *Trends Cell Biol 18*, 552-559.
258. Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A., and Obeid, L. M. (1995) Role of ceramide in cellular senescence, *J Biol Chem 270*, 30701-30708.
259. Sonnino, S., Cantu, L., Corti, M., Acquotti, D., and Venerando, B. (1994) Aggregative properties of gangliosides in solution, *Chem Phys Lipids 71*, 21-45.