Chapter 17
Glycosphingolipid Disorders of the Brain

Stephanie D. Boomkamp and Terry D. Butters

Abstract Glycosphingolipids, comprising a ceramide lipid backbone linked to one/more saccharides, are particularly abundant on the outer leaflet of the eukaryotic plasma membrane and play a role in a wide variety of essential cellular processes. Biosynthesis and subsequently degradation of these lipids is tightly regulated via the involvement of numerous enzymes, and failure of an enzyme to participate in the metabolism results in storage of the enzyme's substrate, giving rise to a lysosomal storage disease. The characteristics, severity and onset of the disease are dependent on the enzyme deficient and the residual activity. Most lysosomal storage disorders found thus far are caused by a defect in the catabolic activity of a hydrolase, causing progressive accumulation of its substrate, predominantly in the lysosome. Storage of gangliosides, sialic acid containing glycosphingolipids, mostly found in the central nervous system, is a hallmark of neuronopathic forms of the disease, that include GM1 and GM2 gangliosidoses, Gaucher type II and III and Niemann-Pick C. Models for these diseases have provided valuable insight into the disease pathology and potential treatment methods.

Treatment of these rare but severe disorders proves challenging due to restricted access of therapeutics through the blood-brain barrier. However, recent advances in enzyme replacement, bone marrow transplantation, gene transfer, substrate reduction and chaperon-mediated therapy provide great potential in treating these devastating disorders.

Keywords Glycosphingolipid · imino sugar · lysosomal storage disease · neurodegeneration · therapeutic strategies

Abbreviations CBE: conduritol B-epoxide; CSF: cerebrospinal fluide; CMT: chaperon-mediated therapy; CNS: central nervous system; ER: endoplasmic reticulum; ERAD: endoplasmic reticulum associated degradation; ERT:

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enzyme replacement therapy; GalNAc: *N*-acetylgalactosamine; GalNAcT: *N*-acetylgalactosaminyltransferase; GA2: gangliotriglycosylceramide; Gb3: globotriaosylceramide; GlcCer: glucosylceramide; GlcNAc: *N*-acetylglucosamine; GM1a: II^3-α-N-acetylneuraminylgangliotetraglycosylceramide; GcGM1a: II^3-α-N-glycolylneuraminylgangliotetraglycosylceramide; GM2: II^3-α-*N*-acetylneuraminylgangliotriglycosylceramide; GM3: II^3-α-N-acetylneuraminyllactosylceramide; GM2AP: GM2 activator protein; GSL(s): glycosphingolipid(s); Hex: hexosaminidase; IL, interleukin; LacCer: lactosylceramide; LDL: low density lipoprotein; LSD(s): lysosomal storage disease(s); M-CSF: macrophage colony stimulating factor; MCB: membranous cytoplasmic body; MHC: major histocompatibility complex; MIP-1α: macrophage inflammatory protein-1α; NeuAc: sialic acid; *N*B-DGJ: *N*-butyldeoxygalactonojirimycin; *N*B-DNJ: *N*-butyldeoxynojirimycin; NPC: Niemann-Pick C; OS: oligosaccharide; SAP(s): sphingolipid activator protein(s); SRT: substrate reduction therapy; TGF-β1: transforming growth factor-β1; TNF-α: tumour necrosis factor-α; TNFR1: tumour necrosis factor receptor 1.

17.1 Introduction to Glycosphingolipid Metabolism and Disease

All eukaryotic cells contain a lipid outer membrane composed of glycerolipids, sphingolipids and sterols. All three lipids have been found to exhibit a wide range of combinatorial diversity and their biochemical and biophysical properties determine functionality.

The backbone of all sphingolipids from which the name is derived, is the sphingoid long-chain base. The most common of these lipids are sphinganine and sphingosine. Ceramide, the simplest sphingolipid and the common precursor for more complex lipids, consists of sphingosine to which a fatty acid is attached. Coupling of a glucose or galactose monosaccharide to ceramide is the first step in the formation of glycosphingolipids (GSLs). GSLs are the most structurally diverse sphingolipids and carry out an enormous range of essential cellular functions. In order to maintain the integrity of the cell, GSLs are continuously synthesised in the endoplasmic reticulum (ER) and the Golgi apparatus, and degraded in the lysosome. If there is a metabolic deficiency in the enzyme required for the degradation of a GSL, substrate accumulates to pathological levels, giving rise to a lysosomal storage disease (LSD). Therapy for treating these relatively rare but severe disorders proves challenging, especially when the nervous system is affected.

17.2 Metabolism

Glycosphingolipids are ubiquitous components of all eukaryotic plasma cell membranes and are particularly abundant at the cell surface. GSLs and their metabolites have been shown to act as intracellular signalling molecules that

modulate numerous essential processes such as cell-cell interaction, proliferation, differentiation, cell death and stress response dependent on cell-type (Zeller and Marchase, 1992).

From our knowledge of human disease states, it can be speculated that GSLs are required for at least one stage of human embryogenesis, as there are no diseases resulting from mutations encoding enzymes involved in the initial steps in the GSL biosynthetic pathway. Knock-out mice that lack ceramide glucosyltransferase, the first enzyme required for GSL biosynthesis, die *in utero* due to widespread apoptosis (Yamashita et al., 1999). Besides playing a vital role in mammalian embryonic development and cellular differentiation, GSLs have also been found to play a crucial role in spermatogenesis (Sandhoff et al., 2005).

Other genetically engineered mouse models deficient in specific genes encoding GSL biosynthetic enzymes ß-1,4-*N*-acetylgalactosaminyltransferase and/or GD3 synthase, are viable despite the absence of complex gangliosides and do exhibit neurological abnormalities (Proia, 2003). Thus, gangliosides are essential for the stabilisation of the central nervous system (Yamashita et al., 2005).

The wide variety of cellular functions demonstrates that GSLs are not merely structural components of the plasma membrane and in order to maintain the integrity of the cell, the lipid bilayer is in a state of constant remodelling, and therefore GSLs are synthesised and degraded continuously.

Biosynthesis of glycolipids, and subsequently GSLs, commences in the ER with the formation of a long-chain aliphatic amino alcohol, sphingosine. This reaction is catalysed by the enzyme serine palmitoyl transferase via two steps, coupling of palmitoyl CoA and serine, forming 3-ketosphinganine, followed by subsequent acylation to form *N*-acyl sphingosine and ceramide when various fatty acids are linked to the 2-amino group of sphingosine (Fig. 17.1).

Hereafter, the *de novo* synthesised ceramide is translocated to the Golgi apparatus where stepwise glycosylation takes place. The mechanism of transport from the ER to the Golgi has been studied extensively but yet remains unclear, and vesicular membrane flow as well as non-vesicular transport has been described in the literature (van Meer and Lisman, 2002).

Coupling of a glucose residue to ceramide by glucosyltransferase gives rise to glucosylceramide (GlcCer), which can then be converted to lactosylceramide (LacCer), the common precursor for all GSLs.

The sequential addition of further monosaccharides and sialic acid residues by specific glycosyltransferases generates the manifold members of the ganglioside series. The newly synthesized GSLs are then transported to the plasma membrane via exocytotic membrane flow, where they carry out a wide variety of cell-type dependent functions.

It has been estimated that as much as 90% of cell GSLs are synthesised after endocytosis of their precursors (Gillard et al., 1998). This process can be disrupted via administration of *N*-alkylated imino sugars e.g. *N*-butyldeoxynojirimycin (*N*B-DNJ) or its galactose analogue, *N*-butyldeoxygalactonojirimycin (*N*B-DGJ), which inhibit the key enzyme involved in the GSL biosynthetic pathway, ceramide glucosyltransferase, and is discussed in further detail later (see Section 17.5.2).

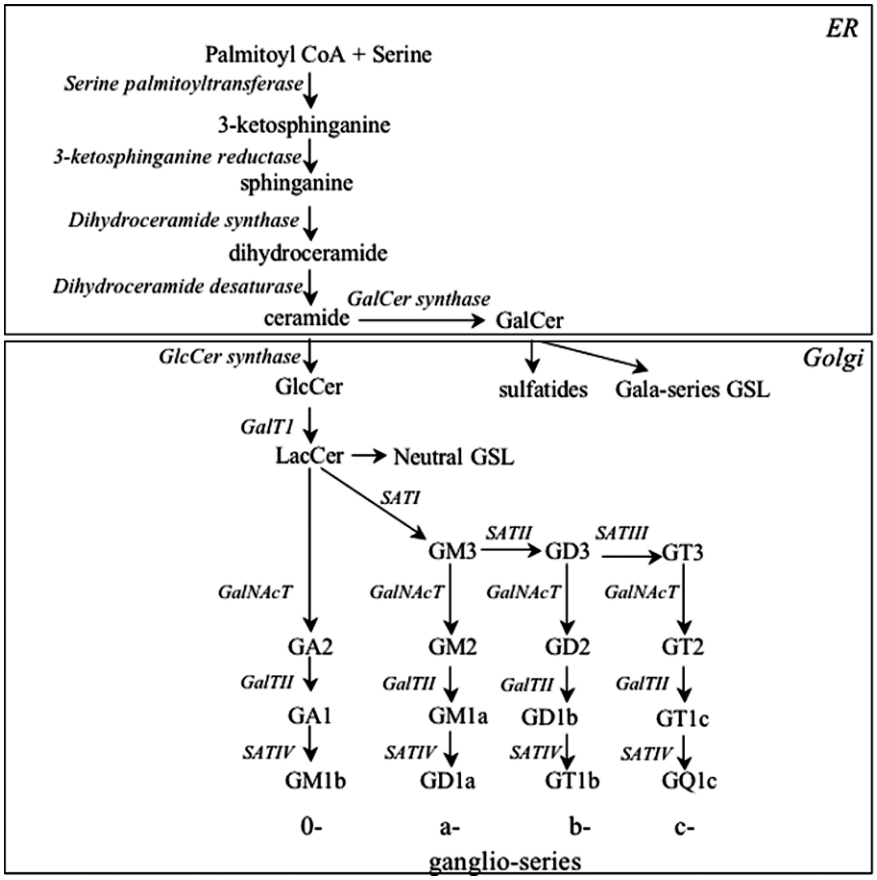

Fig. 17.1 Synthesis of sphingolipids in the endoplasmic reticulum (ER) and the Golgi apparatus. GalNAcT: *N*-acetylgalactosamine transferase, GalT: galactosyltransferase, SAT: sialyl transferase

GSLs can be recycled in the Golgi and possibly the ER where further monosaccharides are added, or endocytosed and trafficked through the endosomal compartments to the lysosome for degradation to more simple metabolites.

Degradation is essentially the reverse of biosynthesis, taking place in the lysosome, discovered by de Duve and colleagues in 1955 (De Duve et al., 1955). GSLs are degraded by water-soluble exoglycosidases, present in the lumen of the lysosome, that sequentially cleave the oligosaccharide residues to produce ceramide, which then undergoes deacylation to produce sphingosine. This lipid can then leave the lysosome and re-enter the biosynthetic pathway or be degraded further. Besides exohydrolases, sphingolipid activator proteins (SAPs) are required for the degradation of sphingolipids with short hydrophilic

head groups. SAPs perturb the membrane and bind the lipid in order to present it to the soluble enzyme for digestion (Kolter and Sandhoff, 2005).

If there is an inherited defect in SAP or a lysosomal glycosidase, GSL accumulates within the lysosome upstream of the defective reactions, resulting in progressive disease pathology.

To date, most of the diseases resulting from defective GSL metabolism have been found to affect GSL degradation, and only GM3 synthase deficiency has been described thus far that affects GSL biosynthesis.

17.3 Defects in GSL Metabolism

17.3.1 Defects in GSL Biosynthesis: GM3 Synthase Deficiency

Only one disease caused by a biosynthetic defect has been reported so far, GM3 synthase deficiency. An infantile-onset form of epilepsy, developmental stagnation, tonic-clonic seizures and blindness characterize this inherited disorder, and the underlying cause is a nonsense 649C→T substitution mutation in exon 8 of the SIAT9 gene on chromosome 2. This gene encodes GM3 synthase and the mutation results in premature termination of the production of this enzyme and thus a non-functional protein product (Proia, 2004; Simpson et al., 2004). GM3 synthase synthesizes ganglioside GM3 from LacCer, the first step in the synthesis of complex a- and b-series ganglioside species. It remains unknown whether this disease is caused by decreased levels of GM3 or other downstream gangliosides, or by accumulation of LacCer caused by lack of flux through the a- and b-series ganglioside pathways and/or by accumulation of the globoside or isogloboside 0-series via β1,3-*N*-acetylgalactosaminyltransferase (Fig. 17.2).

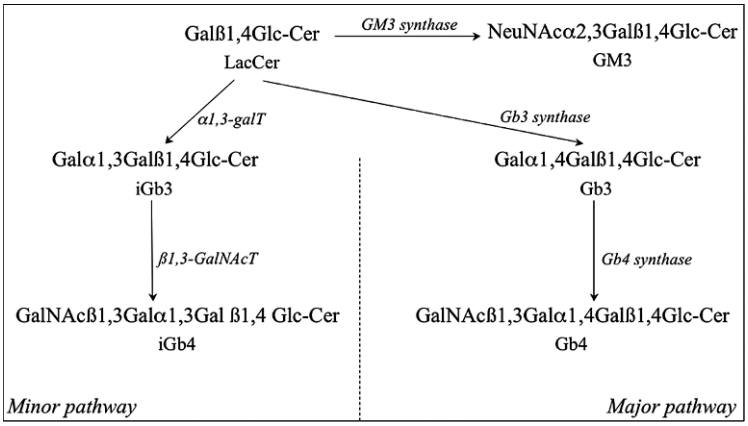

Fig. 17.2 Overview of the biosynthetic pathway of globo-series neutral GSLs and gangliosides. α1,3-galT: α1,3-galactosyltransferase; β1,3-GalNAcT: β1,3-*N*-acetylgalactosaminyltransferase

GM3 synthase is a ubiquitously expressed protein present at particularly high levels in the central nervous system (CNS). The generation of a genetically engineered GM3 synthase knock-out mouse model led to the further understanding of the function of its product, GM3 ganglioside. It was found that these mice exhibited increased insulin sensitivity, suggesting GM3 as a negative regulator of insulin signalling and thus a potential therapeutic target for treating type II diabetes (Aerts et al., 2007).

Intriguingly however, this mouse model was phenotypically normal in contrast to the severe epileptic and developmental phenotype in humans; the reason as to how the pathological mechanisms differ amongst species is at present unclear (Yamashita et al., 2003).

Furthermore, in metastatic GM3 synthase deficient R3230AC cells the isogloboside (iGb4Cer) level is dramatically increased, suggesting that the presence of GM3 synthase prevents the formation of this metastasis-associated glycolipid in these cells. Therefore, GM3 synthase may play a crucial role in the progression of malignant cancer (Dumonceaux and Carlsen, 2001).

17.3.2 Defects in GSL Degradation

In contrast to defects in glycolipid synthesis, diseases associated with defective degradation have been studied extensively and more than 40 lysosomal storage diseases are known of which at least ten are due to defective sphingolipid degradation (Table 17.1) (Futerman and van Meer, 2004). The frequency of individual diseases is not high but collectively they are a significant and severe group of disorders with a frequency of 1:7,700 live births in Australia and the most common cause of paediatric neurodegenerative diseases (Meikle et al., 1999). Some LSDs are more prevalent among specific ethnic groups, such as the high incidence of Tay-Sachs disease (1/3,900 compared to 1/200,000 in the general population) (Petersen et al., 1983) and Gaucher disease (1/855 compared to 1/100,000) among Ashkenazi Jews (Zimran, 1997).

The majority of LSDs are autosomal recessive inherited, with the exception of X-linked Fabry disease.

LSDs are normally classified according to the type of substrate that accumulates. Interestingly however, GSLs, such as GM2 and GM3 ganglioside, may also accumulate secondarily to accumulation of primary storage materials, as seen in Niemann-Pick disease. This additional accumulation, predominantly observed in axons of neurons and ectopic dendrites is essentially identical to that observed in the gangliosidoses (Walkley, 2004). Despite the underlying severe lesion in NPC does not necessarily involve defects in ganglioside metabolism, neuronal function in this LSD is impaired due to secondary storage of GSLs. The mechanism behind the alteration of these gangliosides in many types of lysosomal storage remains elusive.

Table 17.1 Deficiencies and storage products in LSDs

LSD	Enzyme/protein deficiency	Primary storage	Secondary storage
Farber*	Ceramidase	Ceramide	
Fucosidosis*	α-Fucosidase	Pentahexosylfuco-glycolipid	
Sialidosis* (mucolipidosis I)	Sialidase	GM3, sialyl-OS, sialoglycoproteins	GD3, GM4, LM1
Metachromatic leukodystrophy*	Arylsulfatase A or saposin B	Cerebroside sulfate, 3-0-sulfogalactosyl-containing glycolipids	
Galactosialidosis*	Protective protein/ cathepsin A	Sialyl-OS	GM2, GM3, GM1, GD1a
Niemann-Pick			
A*, B	Sphingomyelinase	Sphingomyelin	GM2, GM3
C*	Mutation in NPC1 or NPC2 gene	Cholesterol, bismono-acylglycerol phosphate	GM2, GM3, (GM1)
Krabbe*	Galactosylceramidase	Galactosylceramide	
Gaucher I, II* and III*	ß-glucocerebrosidase	GlcCer	GM2, GM3, GM1, GD3
Fabry	β-galactosidase A	Gb3, Digalacto-sylceramide	
GM1 gangliosidosis*	ß-galactosidase	GM1, GA1, Gal-OS	GM2, GM3
GM2 gangliosidosis*:			
Tay-Sachs	ß-hex A	GM2, GlcNAc-OS	Phospholipids, cholesterol
Sandhoff	ß-hex A, B	GM2, GA2, GlcNAc-OS	
GM2 activator	GM2 activator protein	GM2	

* Neurological involvement
Lysosomal storage diseases, their deficiency, primary storage, and secondary storage products are shown. OS: oligosaccharide.

Despite the different types of primary substrate that accumulate in different LSDs, all LSDs to date are characterized by macrophage activation following storage of GSLs by phagocytosis of senescent and apoptotic cells, and disruption of endocytic pathways targeted to the Golgi. However, the disease pathology, onset and severity are dependent on the enzyme deficiency and residual enzyme activity. Thus, every LSD exhibits pathological features characteristic for that particular disorder.

In this chapter the focus will be on the most common LSDs with neurological involvement, Niemann-Pick type C, types II and III Gaucher disease, GM1 and GM2 gangliosidosis.

17.3.2.1 GM2 Gangliosidoses

GM2 gangliosidosis is a family of autosomal recessive disorders characterized by accumulation of GM2 ganglioside and its related glycolipids in the neuronal lysosome. It comprises GM2 activator protein deficiency, Tay-Sachs and Sandhoff disease, the latter of which are caused by a deficiency in the α-subunit or ß-subunit of ß-hexosaminidase, respectively (Gravel et al., 1995).

Sandhoff disease is a LSD that arises from a variety of point/substitution mutations in the HexB gene on chromosome 5 which encodes the ß-subunit present in ß-hexosaminidase A (a heterodimer consisting of an α- and ß-subunit) and B (ßß). ß-Hexosaminidase is a lysosomal enzyme required for the hydrolysis of the ß-glycosidic links of *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc) terminating glycoconjugates.

Neutral substrates such as glycolipid GA2 can be catabolised by ß-hexosaminidase B as well as A, whereas only the α-subunit is able to hydrolyse negatively charged substrates, e.g. GM2 ganglioside, in the presence of its substrate-specific cofactor, GM2 activator protein (GM2AP) (Hou et al., 1996). Normally, membrane-bound lysosomal GSLs with short oligosaccharide chains, such as GM2, require a sphingolipid activator protein (SAP), such as GM2AP, to lift it out of the membrane and present it to lumenal ß-hexosaminidase A for degradation (Werth et al., 2001).

The third Hex isozyme, HexS, consisting of two α-subunits, does not occur in high quantities and was not thought to have a high catalytic activity. However, it has been shown that double knock-out mice that are totally deficient in Hex activity have a more severe phenotype than mice expressing only HexS as they display mucopolysaccharidosis (Sango et al., 1996) besides gangliosidosis and accumulate more anionic oligosaccharides. This demonstrates that HexS does contribute to the activity of ß-hexosaminidase, and has the potential to catabolise sulphated glycosaminoglycans, GSLs and water-soluble and amphiphilic glycoconjugates (Hepbildikler et al., 2002).

The deficiency in hexosaminidase A and B occurring in Sandhoff disease results in accumulation of GM2 and GA2 primarily in the lysosomes of neuronal cells (Fig. 17.3), leading to the formation of membranous cytoplasmic bodies (MCBs) (Tutor, 2004).

Furthermore, the levels of free oligosaccharides containing ß-linked *N*-acetylglucosamine at their non-reducing terminus, of which the formation is due to incomplete degradation of glycoproteins, are elevated (Fig. 17.4) (Warner et al., 1985; Winchester, 2005).

The main clinical features of this group of neurodegenerative diseases include startle reaction, hypotonia, psychomotor retardation, blindness and a greatly reduced life span of which the severity and disease onset are dependent on residual enzyme activity (Table 17.2) (Jeyakumar et al., 2002). All forms of acute, infantile GM2 gangliosidosis are characterized by a total absence of ß-hexosaminidase and thus no or hardly any residual enzyme activity, whereas the chronic, juvenile/adult forms have approximately 5%, and asymptomatic

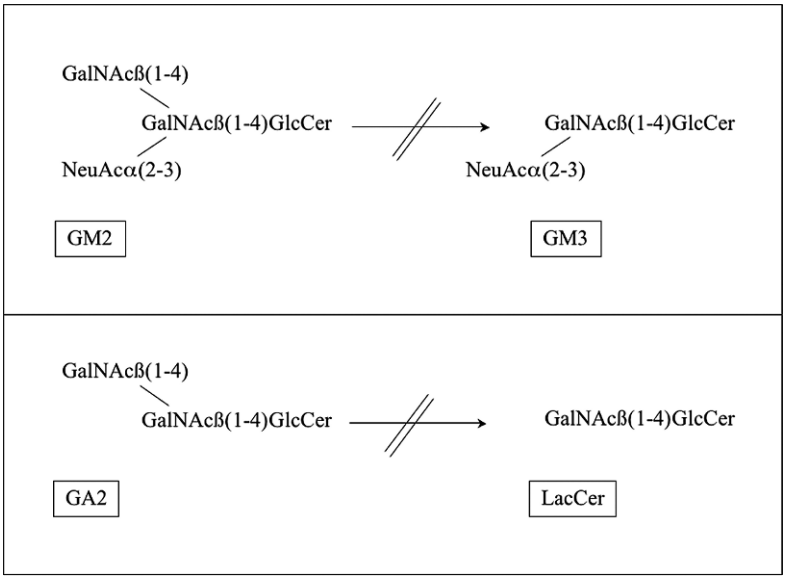

Fig. 17.3 Structures and degradation of GM2 and GA2 glycolipid. In normal cells, *N*-acetylgalactosamine terminating GM2 ganglioside and its asialo-derivative GA2 are hydrolysed to GM3 and lactosylceramide respectively, by hexosaminidase. Deficiencies in hexosaminidase activity results in the accumulation of GM2 and GA2
GalNAc: *N*-acetylgalactosamine; Gal: galactose; Cer: ceramide; NeuAc: sialic acid

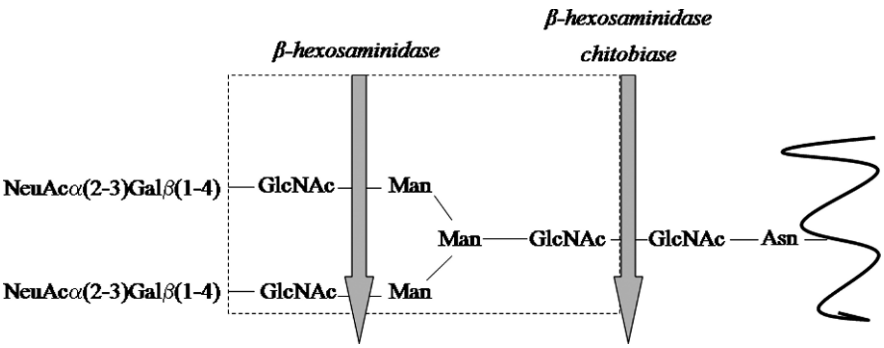

Fig. 17.4 Simplified structure of a complex N-linked glycan and the sites of action of ß-hexosaminidase. Glycoproteins are degraded by specific enzymes from both directions. Lysosomal catabolism is terminated when an *N*-acetylglucosamine residue is encountered by the catalytically impaired enzyme ß-hexosaminidase, and thus the glycoprotein-derived oligosaccharide in the box accumulates in Sandhoff patients.
(NeuAc: sialic acid, Gal: galactose, Man: mannose, Asn: asparagine)

Table 17.2 Major features of GM2 gangliosidoses (adapted from Jeyakumar et al., 2002)

Clinical sign or symptom	Infantile	Juvenile	**Adult**
Motor dysfunction	+ +	+	+
Hypotonia	+ +	+	+
Blindness	+ +	+	–
Seizures	+ +	+	–
Macrocephaly	+	–	–
Cerebellar ataxia	–	+	+
Impaired cognitive function	+	+	+ (–)
Psychosis	–	–	+ (–)
Oligosaccharidurie (Sandhoff)	+	+	?
Organomegaly (Sandhoff)	+	–	–

heterozygotes have been found with just 10% of normal enzyme levels (Tropak et al., 2004).

While the GM2 gangliosidoses have become well understood at the biochemical level, the cause of neurodegeneration remains enigmatic. Initial studies suggested that GM2 ganglioside and/or its derivative may be potential inducers of apoptosis and thus progressive neurodegeneration (Huang et al., 1997). This finding prompted investigators to study the mechanism further, revealing that microglia activation, resulting in the production of inflammatory markers, precedes neuronal cell death (Fig. 17.5).

Bone marrow transplantation of Sandhoff disease mice does not reduce GM2 storage levels in the brain but does lead to the prevention of microglial activation and thus neuronal death (Norflus et al., 1998). Therefore, the inflammatory process may play a key role in the pathogenesis of GM2 gangliosidoses.

Several neurotoxic mediators have been found to contribute to the inflammatory response; for instance, increased tumour necrosis factor-α (TNFα) mRNA levels have been reported in Sandhoff mouse spinal cord, indicating transcriptionally-controlled up-regulation (Wada et al., 2000). Other

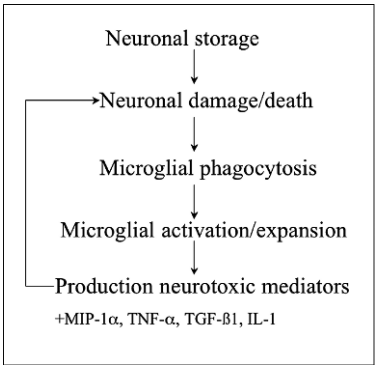

Fig. 17.5 Schematic overview of cellular mechanism in which storage leads to inflammatory response

inflammation markers that have been found to be expressed were major histocompatability complex class II (MHC class II), nitric oxide, interleukin-1ß (IL-1ß) and transforming growth factor-ß1 (TGF-ß1), and their levels rise as the disease progresses. This suggests these markers are produced in response to, or contribute to, the local CNS immune activation (Jeyakumar et al., 2003).

Furthermore, macrophage inflammatory protein-1α (MIP-1α) induction has been suggested to correlate with the accumulation of *N*-acetylhexosaminyl glycoconjugates and not that of GM2 in specific brain regions (Tsuji et al., 2005). This study suggests that accumulation of glycoprotein-derived oligosaccharides as well as that of GM2, and potentially, GA2, may trigger an inflammatory response via different cascades dependent on the storage product. Deletion of this leukocyte chemokine in Sandhoff disease model mice decreases neuronal apoptosis and results in an improved and longer lifespan, further supporting the crucial role of inflammation in neurodegeneration and potentiating the use of anti-inflammatory drugs in the treatment of Sandhoff disease and possibly other LSDs (Wu and Proia, 2004).

17.3.2.2 GM1 Gangliosidosis

GM1 gangliosidosis is an inherited disorder caused by a deficiency in ß-galactosidase, resulting in lysosomal storage of GM1-ganglioside and its asialo-derivative GA1, primarily in the lysosome. Accumulation of these GSLs is widespread, with almost all neurons affected to a certain extent, and gradual deterioration of motor functions. A direct consequence of GM1 accumulation and depletion of ER calcium stores results in the activation of an unfolded protein response, triggering neuronal cell death in the ß-gal$^{-/-}$ mouse model of GM1-gangliosidosis (Tessitore et al., 2004).

A further pathological feature, so far thought to be exclusive to GM1 gangliosidosis, is vacuolisation of visceral cells and in particular liver parenchymal cells, spleen and the glomerular and renal tubular epithelial cells. These vacuoles have been found to contain high levels of highly water-soluble carbohydrate polymers, demonstrating a crucial role of ß-galactosidase in the degradation of these substrates(Wolfe et al., 1974).

Symptom onset and disease severity are dependent on residual enzyme activity, ranging from little or no activity in the infantile and juvenile forms, to measurable activity in adult-onset forms of GM1-gangiosidosis. Infantile-onset patients usually succumb to the disease in the first few months of life.

The potentially contributory role of the accumulating storage products to the pathology of GM1 gangliosidosis has been studied, and the inflammatory process, common to the symptomatic mouse model of GM1 storage appears to pre-date symptom onset, suggesting a potentially contributory role of GM1 in disease progression (Jeyakumar et al., 2003). Compared to wild-type mice, the expression of all apoptosis-mediators studied, MHC class II, Fas and tumour necrosis factor receptor 1 (TNFR1), are significantly elevated in storage regions of the brain, possibly as a result of progressive accumulation of GM1 and its related glycolipids.

The extent of inflammation correlates with disease progression with an age-dependent increase in microglial activation being observed which, interestingly, pre-dates the symptom onset and becomes more extensive as the disease progresses.

Furthermore, local neuroinflammation triggers activation of chemokines, such as stromal-cell-derived factor 1 (SDF-1), macrophage inflammatory protein 1-α (MIP-1α) and MIP-1β. Interestingly, administered β-galactosidase-active bone marrow cells migrate to the central nervous system (CNS), correcting the enzyme deficiency and restoring cytokine and GSL storage levels in GM1 gangliosidosis (Sano et al., 2005a).

17.3.2.3 Gaucher Disease

Gaucher disease is an autosomal recessive progressive disorder and one of the most frequently observed sphingolipidoses. Pancytopenia, hepatosplenomegaly and skeletal complications are hallmarks of Gaucher disease (Zimran, 1997).

The enzyme responsible for degrading glucosylceramide (GlcCer), β-glucocerebrosidase, is deficient which causes progressive accumulation of substrate GlcCer, predominantly in large macrophage-derived cells with a characteristic morphology. These Gaucher cells have been thought to originate from the turn-over of cell membranes, for instance phagocytosed red and white blood cells, and secrete various factors involved in local tissue damage.

In normal situations, hydrolases destined for the lysosome are transported to this acidic compartment via the mannose 6-phosphate receptor. However, recent findings have suggested that β-glucocerebrosidase is trafficked to the lysosome through binding to a transmembrane lysosomal residents protein, LIMP-2, possibly in the endoplasmic reticulum (Reczek et al., 2007). This suggests a novel lysosomal trafficking pathway, independent of the mannose 6-phosphate receptor, and potentially a novel drug target for treating Gaucher disease.

Like many other LSDs, Gaucher disease is remarkably heterogeneous in terms of its clinical expression. Based on neurological involvement, disease onset and thus residual enzyme activity, three subsets can be distinguished, namely non-neuronopathic type I, and acute and sub-acute neuronopathic types II and III, respectively.

Specific mutations in the gene encoding ß-glucocerebrosidase are associated with specific clinical manifestations. The N370S mutation confers type I disease and the L444P mutation often correlates with neurological involvement in type III disease. The neurological manifestations of Gaucher disease have been suggested to be due to the loss of calcium homeostasis related to GlcCer accumulation within neurons (Futerman, 2007).

Recently, it has been shown that Gaucher type I disease can also be caused by a deficiency in Saposin-C, a lysosomal protein required for the degradation of glucosylceramide. Besides GlcCer storage, dramatically increased levels of chitotriosidase, a quantitative marker for the degree of macrophage activation, and chemokine CCL18, highly specific for Gaucher disease, are found in the plasma of Gaucher patients, the levels of which correlate to disease severity. In

contrast, β-glucocerebrosidase activity is normal (Tylki-Szymanska et al., 2007). Accumulation of substrate within Gaucher cells appears to lead to elevation of pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IL-10 and M-CSF, demonstrating direct correlation between GlcCer storage and disease progression (Tylki-Szymanska et al., 2007). Furthermore, the expression of macrophage inflammatory proteins MIP-1α and MIP-1β, both implicated in skeletal complications in multiple myeloma, are significantly elevated (9 and 12 fold respectively) in plasma of Gaucher type I patients. Effective treatment via enzyme replacement (ERT) results in restoration in the levels of these inflammatory markers (van Breemen et al., 2007).

Recent data analysing secondary metabolites also reveal the complexity of this monogenetic disorder that could influence pathogenesis. Macrophage models of Gaucher disease and patient plasma show elevations in ceramide, lactosylceramide, Gb3 and GM3 (Ghauharali-van der Vlugt et al., 2007; Hein et al., 2007). The increase in GM3 in particular may correlate with increased insulin resistance in Gaucher patients (Langeveld et al., 2007).

17.3.2.4 Niemann-Pick Disease

Niemann-Pick disease comprises three subsets, A, B and C, of which the former two are caused by a genetic defect in lysosomal sphingomyelinase activity. Niemann-Pick type C (NPC) however is biochemically distinct from types A and B, due to a mutation in either the NPC1 gene (in more than 90% of the cases) or the NPC2 gene (Sun et al., 2001). The precise functions of NPC1 and NPC2 proteins remain at present unclear; it has been speculated that NPC1 might be implicated in the transport of low density lipoprotein (LDL) derived cholesterol out of the endosomal pathway. NPC2, present in the luminal lysosome, may bind cholesterol and cooperate with NPC1 in mediating the egress of cholesterol from the late endosome to the lysosomal compartment (Zhang et al., 2003).

The clinical manifestations, including vertical gaze palsy, ataxia, dystonia and seizures, usually become evident in early childhood and death typically occurs in adolescence. Although NPC is relatively rare (1 in 150,000 live births), the incidence in Yarmouth County, Nova Scotia, is 1% with an estimated carrier frequency of 10–25% (Winsor and Welch, 1978).

The most characteristic features of the brain tissue of NPC patients are neuronal cell loss, particularly Purkinje cells, swollen neurites and extensive demyelination (Higashi et al., 1993).

The underlying causes of this pathological phenotype have been found to be massive lysosomal accumulation of cholesterol and other lipids including bis-monoacylglyerol phosphate as well as gangliosides GM2 and GM3. The brain is the organ most enriched in cholesterol and as there is no access through the blood-brain barrier, its synthesis takes place in the CNS (Turley et al., 1998). Normally, the majority of the cholesterol in the brain resides in myelin; however in NPC brain tissue cholesterol is absent which is thought to lead to extensive demyelination (Takikita et al., 2004).

Mechanisms leading to pathology in NPC are only beginning to become understood; it has been speculated that the impaired LDL-derived cholesterol from the lysosome in NPC patients results in lysosomal cholesterol sequestration and thus an increase in hydrolases such as cathepsin D. High levels of cathepsin D are found to be toxic and lead to microglial activation (German et al., 2002) and up-regulation of interleukin-1ß confined to degenerating brain regions as a consequence of astroglial activation (Baudry et al., 2003), as well as autophagy (Liao et al., 2007). Autophagy has been found to be involved in neurodegeneration in the to NPC related Alzheimer disease (Nixon et al., 2005). These findings suggest that lysosomal dysfunction indirectly contributes to NPC pathogenesis and its progressive neurodegenerative feature.

17.4 Models for the Glycosphingolipidoses

Relatively recently, various models for sphingolipidoses have been developed in order to elucidate cellular functions of a glycolipid and to study the pathogenesis and potential approaches towards therapy of LSDs. Yeast models, Drosophila, cells obtained from patients, genetically engineered cells and animal models have all provided valuable insight.

An *in vitro* cellular model for Gaucher was developed by treating murine J774 macrophages with irreversible inhibitor conduritol B-epoxide (CBE) and feeding these cells red blood cell ghosts obtained from a Gaucher disease patient, in order to more closely mimic the disease state. CBE inhibits glucocerebrosidase, which is deficient in Gaucher disease. Interestingly, the activity of glucocerebrosidase could be reduced to 11–15% of the normal control level before storage of its substrate glucosylceramide occurred, thus demonstrating that reduction in enzyme activity does not automatically lead to storage and disease pathology (Schueler et al., 2004). This demonstrates that a critical threshold of residual enzyme activity must be reached to cause lysosomal storage (Conzelmann and Sandhoff, 1983).

Furthermore, the use of a similar chemically-derived *in vitro* cellular model led to the therapeutic application of a novel drug, *N*B-DNJ (miglustat, Zavesca®), a potent inhibitor of ceramide glucosyltransferase at a relatively low concentration (5–50 μM) in murine macrophages (Platt et al., 1994) (see Section 17.5.2).

Patient-derived cell lines or those obtained from mouse LSD models have also been used extensively to gain further insights in the disease pathology and potential therapeutics. For example, GM1 gangliosidosis has been studied in patient-derived fibroblasts and those obtained from ß-galactosidase deficient mice. The GM1 synthase activity in human fibroblasts was significantly reduced, whereas in murine fibroblasts the levels were even slightly increased. This suggests the occurrence of alternative metabolic pathways in mouse and man.

Moreover, the use of this model demonstrates that the massive intracellular accumulation of GM1 due to the loss of ß-galactosidase activity affected the biosynthetic pathway of this ganglioside, suggesting the occurrence of

regulatory mechanisms that balance ganglioside biosynthesis and maintain cellular homeostasis (Sano et al., 2005b). Thus, it can be speculated that the turn-over of glycolipids is dependent on residual enzyme activity and therefore GSL metabolism in juvenile/adult patients occurs more rapidly than in infantile patients with no or hardly any activity (Leinekugel et al., 1992).

Genetically engineered mouse models for disease have also been used to develop authentic *in vitro* models. Aortic endothelial cells isolated from Fabry mice, which lack α-galactose A, retain the elevated levels of globotriaosylceramide (Gb3) observed in culture (Shu et al., 2005). This demonstrates that when LSD cells are removed from their native environment, the disease phenotype in terms of storage is still present. Thus, *in vitro* cell models for gangliosidoses are suitable for studying these storage diseases, as the reproducibility of cultured cells compared to multi-cellular organisms is higher due to controlled conditions, and the effects of higher concentrations of potential therapeutic drugs can be tested, as well as the performance of long-term experiments.

Most studies on GM2 gangliosidoses have been performed on mouse knockout models. However, while only the human phenotype slightly differs from that observed in these mice, the severity and disease course differs significantly, as the result of a sialidase, so far thought to be exclusively present in mice (Kolter and Sandhoff, 1998) (Fig. 17.6).

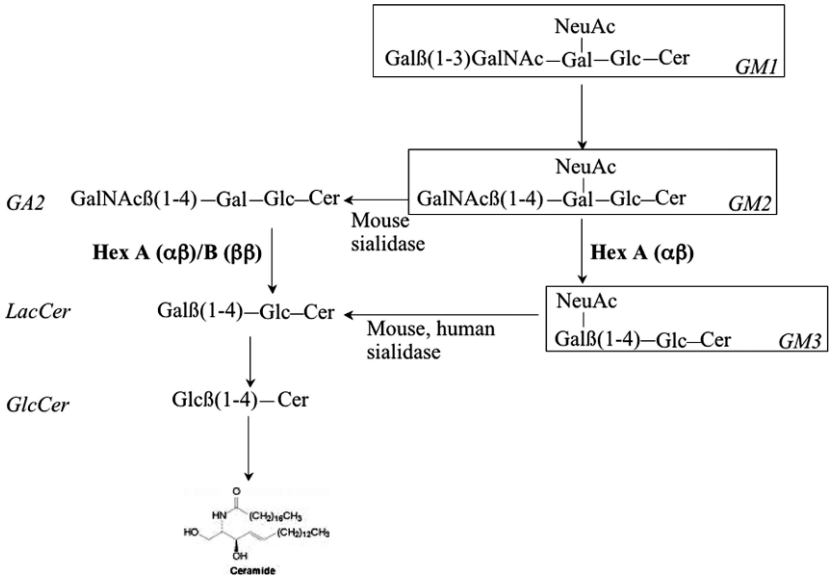

Fig. 17.6 Partial GSL degradation pathway. GM2 is degraded by hexosaminidase A (Hex A) in cooperation with GM2 activator protein to form GM3. The presence of a specific sialidase in mice prevents accumulation of this glycolipid in GM2 gangliosidosis and degrades its substrate to GA2, which in turn can be degraded by Hex A or B. GM3 is acted on by sialidase, present in mouse and human, and lactosylceramide (LacCer) is formed which is then further degraded by specific hydrolases

For example, by contrast to human patients, Tay-Sachs knock-out mice ($Hexa^{-/-}$) show no neurological abnormalities despite the progressive accumulation of GM2 ganglioside in the central nervous system, whereas the Sandhoff knock-outs ($Hexb^{-/-}$) are severely affected (Sango et al., 1995). It has been proposed that this difference of the Tay-Sachs disease severity between mice and humans is due to the possibility that $Hexa^{-/-}$ mice, not humans, escape disease pathology through partial catabolism of GM2 via GA2 which can then be catabolised by active ß-hexosaminidase B (Phaneuf et al., 1996).

In our laboratory an authentic *in vitro* cellular model for Sandhoff disease has been generated upon treatment of RAW264.7 murine macrophages with an inhibitor of β-hexosaminidase (Boomkamp and Butters, unpublished data). High performance liquid chromatography (HPLC) analyses of extracted GSL oligosaccharides show that GA2 predominantly accumulates in inhibitor-treated RAW cells as opposed to a minor but significant increase in GM2 levels (Fig. 17.7). This difference in elevation is possibly due to the

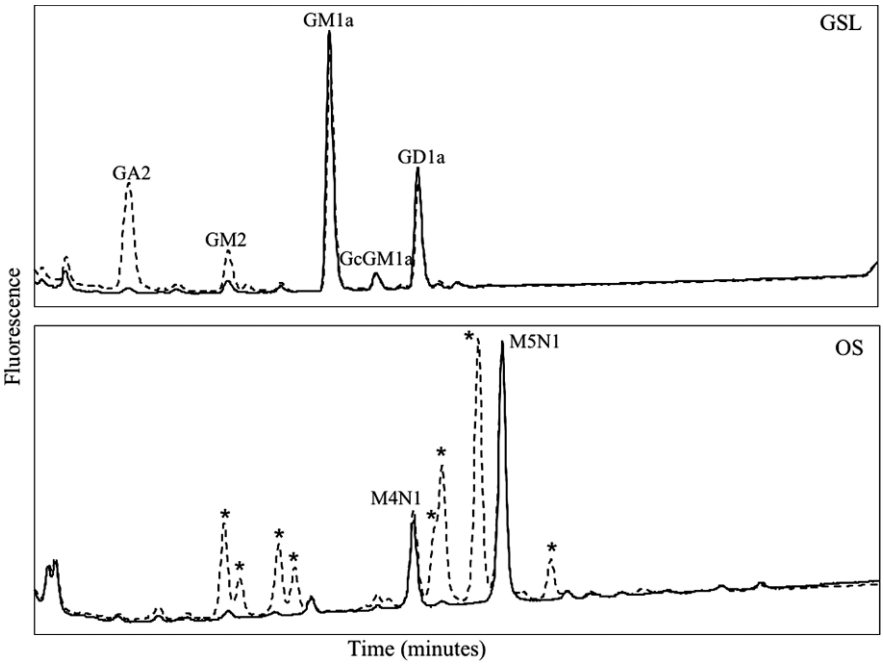

Fig. 17.7 HPLC chromatograms of GSLs and OS in RAW264.7 cells prior and following treatment with ß-hexosaminidase inhibitor.
Top panel: Untreated RAW cells contain mainly GM1a, GcGM1a and GD1a (*solid line*) whereas upon treatment GA2 and GM2 glycolipid are additionally present (*dotted line*).
Bottom panel: Untreated RAW cells contain mainly polymannose M4N1 and M5N1 (*solid line*), whereas upon treatment the levels of GlcNAc-terminating structures (marked with an *asterisk*) are significantly increased (*dotted line*)

presence of a mouse-specific sialidase that provides a bypass mechanism by hydrolysing the sialic acid residue from GM2 to form GA2, as discussed previously.

Furthermore, glycoprotein-derived oligosaccharide (OS) purifications show that inhibitor treated cells exhibit a wide variety of GlcNAc-terminating structures, in addition to polymannose-type structures $Man_4GlcNAc_1$ (M4N1) and $Man_4GlcNAc_1$ (M5N1), also present in untreated cells which are products of ER associated degradation (Alonzi et al., 2008) (Fig. 17.7).

In urine and tissues of Sandhoff patients, OS are observed which serve as biological markers for disease. The main species, accounting for 70% of the oligosaccharides stored in the brain, has been shown to be a biantennary, bisected heptasaccharide, $Man_3GlcNAc_4$, (Warner et al., 1985) consistent with that seen in the Sandhoff mouse model (Lowe et al., 2005). This structure is also a major species in inhibitor-treated RAW cells. All other stored OS were also found to be GlcNAc-terminating, illustrating the deficiency/inhibition of ß-hexosaminidase and termination of further degradation of the partially hydrolysed glycoprotein.

Overall, the developed *in vitro* RAW model is an authentic, cellular representative of Sandhoff disease and may provide further insights into the disease pathology and for evaluating potential therapies for this disorder.

17.5 Therapeutic Options for the Neuronopathic Glycosphingolipidoses

Until recently few treatment options were available for LSDs with neurological involvement and the progressive nature of disease pathology in the most severe phenotypes allowed little time for clinical intervention. For those patients with juvenile and late-onset disease the potential for slowing the course of disease should have a dramatic outcome on quality of life measurements but the prospects for generating therapeutics for orphan diseases, where the population of affected individuals are extremely low, appeared to be poor. However, the success of enzyme supplementation for type 1 Gaucher disease (Brady, 2006) has provided a platform for pre-clinical studies for treating similar glycosphingolipid storage disorders.

The approaches to disease intervention can be separated into an enzyme augmentation strategy that includes direct enzyme infusion, pharmacological chaperoning, bone marrow replacement and gene transfer, and inhibition of substrate synthesis (Butters, 2007b). The first approach is dedicated to the precise enzyme deficiency in each disease whereas substrate inhibition is a generic therapy that can be applied to all glycosphingolipidoses that have a common biosynthetic pathway.

17.5.1 Enzyme Augmentation

17.5.1.1 Enzyme Replacement Therapy (ERT)

Improving the catalytic competence in the lysosome can be achieved by direct infusion of an enzyme to provide enzyme augmentation or enzyme replacement therapy. For those diseases where neurological storage of the products of incomplete metabolism is the major biochemical phenotype, such as the gangliosidoses and types II and III Gaucher disease (Table 17.1), the blood-brain barrier restricts the access of intravenously delivered protein and results in a less efficient outcome for reducing the storage burden. Some success has been achieved by direct intracerebroventricular injection or high dose infusion (Vogler et al., 2005) but the clinical response may be insufficient to provide benefit.

17.5.1.2 Molecular Chaperons

In many of the glycosphingolipidoses, amino acid changes as a result of gene mutations cause the protein to misfold during translation in the ER. Some of this protein is removed from the ER by a process of ER-associated degradation (ERAD) and other misfolded variants may be inefficiently trafficked to the lysosome (Ron and Horowitz, 2005). The net result is a reduction in lysosomal catalytic activity. The use of small molecules as chaperons to protect the protein from gross misfolding and ERAD, enhances the catalytic activity of enzyme in the lysosome. This novel approach, chaperon-mediated therapy (CMT), is in the early stages of clinical evaluation for the peripherally associated storage disorders, such as Gaucher and Fabry disease, following key pre-clinical studies (Butters, 2007b; Butters et al., 2005; Fan, 2003; Pastores and Sathe, 2006).

Many of the chaperons used have been imino sugars because of their tight-binding properties to the active site and at sub-inhibitory concentrations produce an enhancement of enzyme that may be sufficient to degrade the lysosomally stored material. For the neuronopathic disorders few candidates have been identified but a recent chemical library screen for potential hexosaminidase chaperons has revealed additional molecular frameworks that could be used that are outside the imino sugar structural motif (Tropak et al., 2007).

In the glycosphingolipidoses, the severity of disease is determined by the site(s) of mutation that critically affect protein folding. Whilst the mild to moderate disorders of protein folding, such as Gaucher and Fabry disease may be amenable to CMT, the more severe phenotypes may pose difficulty. In Gaucher disease, a common mutation predicting a neuronopathic course of disease is L444P, and the lack of ability of imino sugar chaperons to rescue enzyme activity and provide therapeutic potential may be limited (Butters, 2007a). Despite this, the identification of small molecules such as the imino sugars used for substrate reduction therapy (SRT) have promise since the blood-brain barrier is crossed, although rather inefficiently, and many of the safety and toxicity concerns for using these as pharmacological

agents in man have been allayed. At the experimental level, partial correction of the cellular activity of the misfolded enzyme has been achieved for Gaucher ß-glucocerebrosidase (Compain et al., 2006; Sawkar et al., 2005; Steet et al., 2006; Yu et al., 2007; Zhu et al., 2005), Fabry α-galactosidase (Asano et al., 2000; Fan, 2003; Yam et al., 2005), GM1 gangliosidosis (Matsuda et al., 2003; Suzuki, 2006; Tominaga et al., 2001) and Tay-Sachs/Sandhoff ß-hexosaminidase (Tropak et al., 2004).

17.5.1.3 Bone Marrow Transplantation and Gene Therapy

In mouse models for the gangliosidoses, bone marrow transplantation (BMT) extended the life span, slowed the progressive neurological deficit and reduced the peripheral cellular storage of glycoconjugates (Norflus et al., 1998). The extent to which donor-derived macrophages are able to repopulate the CNS and cross-correct the enzyme deficiency may be a limitation for this approach for severe disorders. A combination of approaches may be more successful and a synergistic improvement in the survival rate was demonstrated in the Sandhoff mouse when BMT was used in conjunction with SRT (Jeyakumar et al., 2001). In man, transplantation carries a number of risks associated with an invasive technique but offers a longer-term solution to enzyme infusion. The relatively few clinical reports suggest that this may be an option when other therapeutic approaches are unavailable.

Gene therapy is still at the pre-clinical stage and cellular and *in vivo* studies (Guidotti et al., 1998; Kyrkanides et al., 2005) support the utility of this approach for cross-correction of cells in privileged sites such as the brain. In the Sandhoff mouse model, enhanced survival following delivery to the brain via stereotaxic injection of adeno-associated viral vectors (Cachon-Gonzalez et al., 2006) was observed to be greater than a combination of BMT and SRT (Jeyakumar et al., 2001).

17.5.2 Substrate Reduction Therapy (SRT)

Substrate reduction therapy is a strategy that aims to partially inhibit the biosynthetic cycle to reduce glycosphingolipid substrate influx into the catabolically compromised lysosome. A number of small molecules have been synthesised to inhibit ceramide-specific glucosyltransferase, the first enzyme in the biosynthetic pathway that glycosylates ceramide lipid, to balance synthesis with catabolism and restore homeostasis. *N*B-DNJ was shown to be an effective inhibitor of glycolipid biosynthesis and could reduce lysosomal storage in a chemically-derived tissue culture cell model of Gaucher disease (Platt et al., 1994). In mouse, models for disease were generated by deleting the gene encoding for lysosomal hexosaminidase and SRT using *N*B-DNJ was demonstrated to have biochemical efficacy in reducing neuronal cell

ganglioside in the Tay-Sachs mouse (Platt et al., 1997) and improved the life span by 40% in the Sandhoff mouse (Jeyakumar et al., 1999). These data allowed a proof of principle study of *N*B-DNJ (miglustat) in man, and a 12-month assessment for efficacy in type I Gaucher disease showed improvements of organ volumes and haematological parameters (Cox et al., 2000). Miglustat gained approval in Europe, USA and Israel for use for type I patients who were unable or unwilling to take ERT. Clinical benefit was clearly demonstrated in this multi-centre trial and further trials to evaluate low dose administration (Heitner et al., 2002) and a 3-year continuation study (Elstein et al., 2004) have revealed significant improvements in all the major clinical endpoints. These data are consistent with the mechanism of action of miglustat as a drug dependent modulator of GSL biosynthesis since treatment was increasingly effective with time, as confirmed by further studies over 24 months (Pastores et al., 2005) and in combination with ERT (Elstein et al., 2007). Additional studies have demonstrated that when miglustat was administered for 12 months to a Spanish cohort of 25 patients, an equivalent improvement in hematologic parameters to ERT was observed (Giraldo et al., 2006). The access of small molecules such as miglustat to tissues that would be intractable to enzyme therapy, such as bone, results in an improvement in bone density and a reduction in pain (Pastores et al., 2007). A significant body of data now supports the use of this drug for SRT in Gaucher disease and other glycosphingolipidoses (Butters, 2007b).

In Niemann-Pick type C disease, where glycosphingolipid storage is a consequence of abnormal lipid trafficking and may contribute to the pathology observed, SRT has been shown to reduce biochemical markers of the lysosomal burden (Lachmann et al., 2004). In two recent studies, miglustat has been shown to stabilize the systemic disease in children with NPC (Chien et al., 2007; Patterson et al., 2007), implying that a reduction in CNS storage of glycosphingolipid can be of therapeutic value in this and similar neurodegenerative disorders.

17.6 Problems and Perspectives

Poor access via the blood-brain barrier is the major obstacle that prevents ERT from being applicable to the neuronopathic disorders. The inflammatory cascade that is mediated by CNS storage seen in the gangliosidoses for example, may respond to steroid treatment but unless the neuronal cell lysosomal burden can be reduced, this peripheral and neurological component of the phenotype could dominate the clinical outcome of disease.

Small molecule therapeutics are generally more able to access the neural tissue and the efficacy of miglustat to reduce neuronal cell ganglioside accumulation in murine models of Tay-Sachs and Sandhoff disease, indicates that a proportion of the plasma dose reaches the CNS. Direct measurement of

miglustat in the cerebrospinal fluid (CSF) obtained from patients with either Niemann-Pick type C disease or Tay-Sachs disease following oral treatment have been obtained. These data reveal that 16–27% of the plasma concentration can be measured in the CSF at an oral dose of 100 mg/day (Bembi et al., 2006; Lachmann et al., 2004). Although the clinical response to treatment is variable and difficult to assess, as with many other progressive neurodegenerative disorders, the expected reduction in lipid burden offers potential for treating those disorders such as Gaucher type II/III, NP-C and the gangliosidoses, where no current successful therapy is available. Although the mechanism of action of miglustat is consistent with pre-clinical data showing a reduction of substrate, data in support of clinical efficacy for treatment of severe neuronopathic disorders is required. Recently, a two year study of a combination of ERT and miglustat administered to 20 type III Gaucher disease patients, aged between two and 20 years, did not demonstrate any improvement of the primary end-points (Butters, 2007a). The inability to reverse much of the existing pathology may be an overriding factor for treating many of the neuronopathic conditions, particularly for paediatric patients who may require high doses of a pharmacological agent to provide a more rapid response. The development of drugs for SRT or CMT with an improved efficacy and safety profile to allow either mono-therapy or in combination at doses required to deplete substrate and/or enhance enzyme activity significantly, may be a partial solution to these problems.

The dissection of the biochemical pathways that generate lysosomal storage of glycoconjugates has been a major achievement but our further understanding to allow the development of novel routes for intervention remains a challenge.

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