Chemical biology of glucosylceramide metabolism fundamental studies and applications for Gaucher disease

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Metabolic adaptations to defective lysosomal glycosphingolipid degradation

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Based on

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Abstract

The cellular recycling of glycosphingolipids (GSLs) to their building blocks is completed in lysosomes and involves the local hydrolytic action of specific glycosidases. Severe reduction of capacity in one of the down-stream steps in degradation of GSLs in lysosomes should result in progressive and prominent accumulation of the corresponding substrate since turnover of endogenous and endocytosed exogenous GSLs is chronic and considerable. The most common inherited deficiencies in lysosomal GSL catabolism are Gaucher disease (GD) and Fabry disease (FD) caused by defects in lysosomal glucocerebrosidase (GBA) and alpha-galactosidase A (GLA), respectively. The accumulation in cells and tissues of glucosylceramide (GlcCer) in GD and that of globotriaosylceramide (Gb3) in FD tends to level with age, suggesting the existence of biochemical adaptations to the primary defects. These poorly appreciated metabolic adaptations, in and beyond lysosomes, are reviewed here. One important adaptation is the de-acylation of accumulating GSLs in lysosomes by the action of the enzyme acid ceramidase. Thus, the lysosomal storage of GlcCer in GD and that of Gb3 in FD is limited through formation of glucosylsphingosine (GlcSph) and globotriaosylsphingosine (lysoGb3), respectively. In the case of GD, another adaptation in metabolism takes place beyond the lysosome, involving the enzyme GBA2 located in the cytoplasmic leaflet of membranes of the endoplasmic reticulum and Golgi apparatus. GBA2 allows extra-lysosomal degradation of GlcCer and concomitantly generates glucosylated cholesterol. The benefit and harm of these metabolic adaptations in GD and FD are discussed.

The life cycle of glycosphingolipids through various subcellular compartments.

The outer leaflet of the plasma membrane is rich in glycosphingolipids (GSLs) with a hydrophobic ceramide (Cer; N-acylated sphingosine) embedded in the lipid layer. Attached to the C1-hydroxyl of Cer are sugars[1,2]. The glycan starts with glucose or galactose and can be further extended by combinations of monosaccharides[1,2]. GSLs interact with cholesterol molecules by van der Waals forces and thus form transient semi-ordered membrane domains, so-called 'lipid rafts'. In these domains specific proteins are preferentially located and related signaling events occur[3–5]. GSLs undergo several chemical modifications during their life in various subcellular compartments (Figure 1). They are *de novo* synthesized starting with the formation of 3-ketosphinganine by the enzyme serine palmitoyltransferase (SPT). At the endoplasmic reticulum it catalyzes the condensation of serine and fatty acyl-CoA with preference for palmitoyl-CoA resulting in 18 carbon sphingoid bases[6]. Next, a specific reductase forms sphinganine that is subsequently metabolized by ceramide synthases (CERS) to dihydroceramides. The enzyme dihydroceramide desaturase (DES) converts these to ceramides[6,7]. The newly formed Cer may be next metabolized in the ER to galactosylceramide (GalCer). Alternatively, Cer is transported by the protein CERT to the cytosolic leaflet of membranes of the *cis*-Golgi apparatus where it is converted to glucosylceramide (GlcCer) by the enzyme glucosylceramide synthase (GCS)[8,9]. Subsequently, translocation of GlcCer to the luminal leaflet of the Golgi membrane may occur via an unknown mechanism whereupon the lipid can be stepwise extended with further sugars by sequential action of glycosyltransferases to complex GSLs like gangliosides and globosides[10,11]. In addition, specific GSLs can be sulfated by sulfotransferases[6]. This metabolism of GSLs and the resulting structural heterogeneity of GSLs is the topic of excellent reviews[1,6,11,12]. From the Golgi apparatus, GSLs move to the outer leaflet of the plasma membrane. GSLs may be exported from the surface of cells through transfer to nascent HDL particles, but most are ultimately internalized. Through endocytosis, GSLs finally end up in multi-vesicular bodies of late endosomes and are subsequently degraded in lysosomes. The degradation of complex GSLs implies sequential removal of terminal sugars by specialized lysosomal glycosidases, often facilitated by specific accessory proteins (GM2 activator protein and saposins A-D)[13]. A common product of GSL fragmentation is Cer, being generated from GlcCer by glucocerebrosidase (GBA) and from GalCer by galactocerebrosidase (GALC). Cer is cleaved in lysosomes by acid ceramidase (AC) to fatty acid and sphingosine ((2S,3R,4E)-2-aminooctadec-4-ene-1,3-diol). The degradation product sphingosine is exported to the cytosol where it may be re-generated to Cer. This salvage pathway is mediated by the CERS enzymes[14]. Alternatively, sphingosine kinases (SK1 and SK2) may convert sphingosine to sphingosine-1-phosphate (S1P) that can be degraded by S1P lysase (SPL) to phosphatidylethanolamine and 2-trans-hexadecenal[15,16].

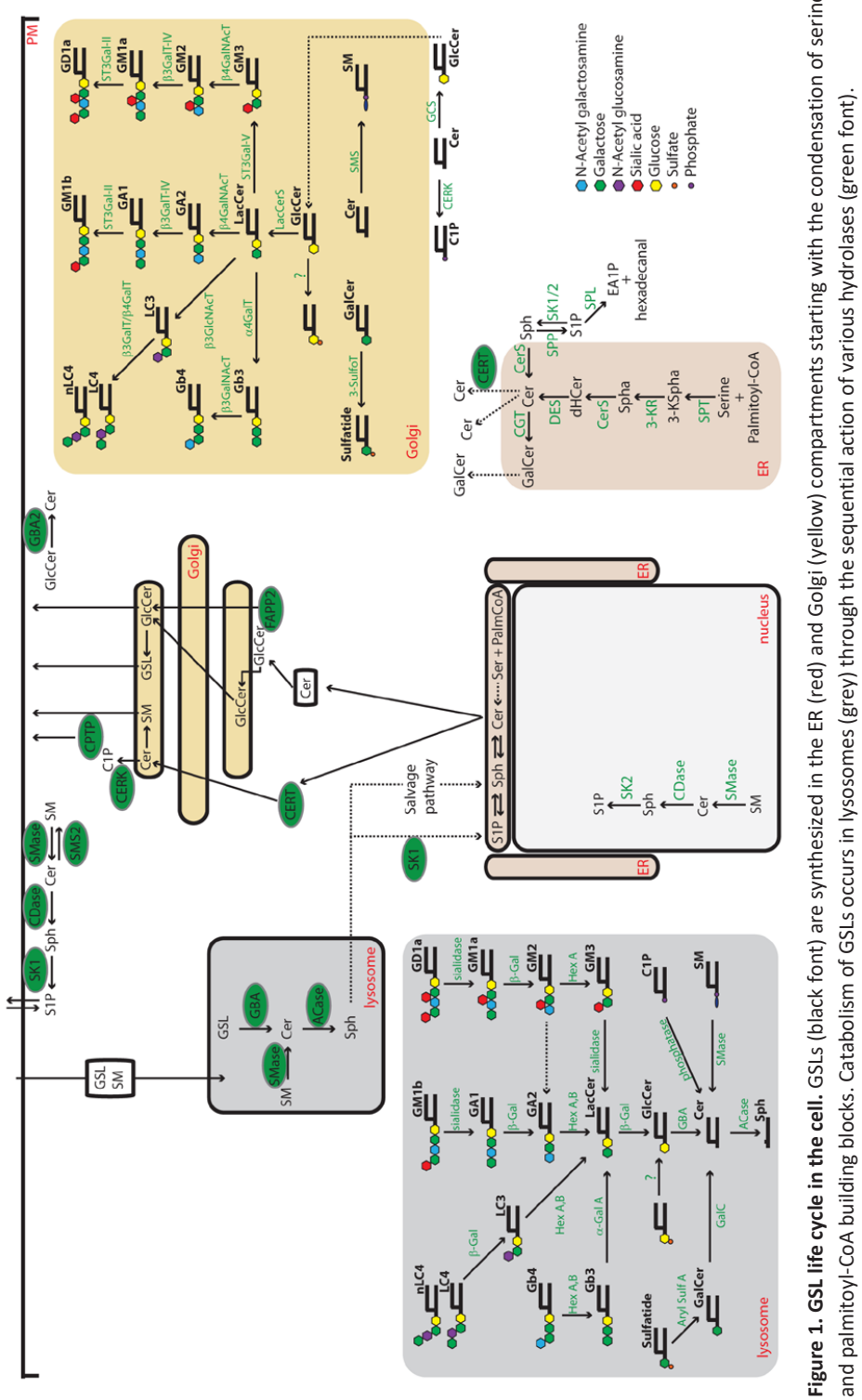


Figure 1. GSL life cycle in the cell. GSLs (black font) are synthesized in the ER (red) and Golgi (yellow) compartments starting with the condensation of serine and palmitoyl-CoA building blocks. Catabolism of GSLs occurs in lysosomes (grey) through the sequential action of various hydrolases (green font).

Impaired lysosomal degradation of GSLs as cause for disease.

A number of inherited deficiencies in lysosomal GSL degradation exist, the so-called glycosphingolipidoses. An inherited disease in man is known for each step in the lysosomal catabolism of the more common glycosphingolipids, except for that of lactosylceramide, (for reviews see references[17–20]). Examples of these disorders are Fabry disease (FD, globotriaosylceramidosis), Gaucher disease (GD, glucosylceramidosis), Krabbe disease (KD, galactosylceramidosis), GM1 gangliosidosis and GM2 gangliosidosis, due to defects in α-galactosidase A, glucocerebrosidase (acid β-glucosidase), galactocerebrosidase, β-galactosidase and β-hexosaminidase, respectively (Table 1). The therapeutic success of supplementing GD patients with lacking enzyme through chronic intravenous infusions (enzyme replacement therapy, ERT) has stimulated similar approaches for other glycosphingolipidoses. This development promoted the screening for individuals with lysosomal glycosidase abnormalities in targeted risk groups as well as newborns[21,22]. The prevalence of the combined glycosphingolipidoses, earlier estimated to be about 1 in 20 000 live births, seems relatively high given the increasingly recognized late onset and atypical variants, particularly for X-linked FD[21].

Table 1. Inherited (glyco)sphingolipidoses.

Disease	*Gene*	*Protein*	*Main storage material*
GM1 gangliosidosis	*GLB1*	Acid β-galactosidase	GM1 ganglioside
GM2 gangliosidosis (Tay-Sachs)	*HEXA*	β-hexosaminidase α subunit	GM2 ganglioside
GM2 gangliosidosis (Sandhoff)	*HEXB*	β-hexosaminidase β subunit	GM2 ganglioside
Fabry	*GLA*	α-galactosidase A	Globotriaosylceramide (Gb3)
Gaucher	*GBA*	Glucocerebrosidase	Glucosylceramide (GlcCer)
Metachromatic leukodystrophy	*ARSA*	Arylsulfatase A	Sulfatide
Krabbe	*GALC*	Galactosylceramidase	Galactosylceramide (GalCer)
Niemann-Pick type A and B	*SMPD1*	Acid sphingomyelinase	Sphingomyelin
Niemann-Pick type C	*NPC1/NPC2*	NPC1/NPC2	Cholesterol, GSLs & sphingomyelin
Farber	*ASAH1*	Acid ceramidase	Ceramide

The *de novo* synthesis and turnover of GSLs is impressive in most cell types with estimated half-lives in the order of hours[6,11]. Cells may furthermore endocytose significant amounts of

GSL-rich lipoproteins. A (near) complete block in lysosomal catabolism should lead to a rapid, and ongoing, accumulation of a GSL in cells, according to the "critical threshold" hypothesis of Conzelmann and Sandhoff that furthermore predicts a linear increase in storage accumulation[23]. Indeed, in glycosidase-deficient cells lysosomal GSL storage generally develops quickly. It already occurs *in utero* with some glycosidase knockout mouse models[24]. However, patients and mice with a glycosphingolipidosis not always show the predicted ongoing GSL buildup: after fast initial accumulation, the subsequent lipid storage increases only marginally. Equally puzzling is the observation that overt disease manifests relatively late in man and mice with complete absence of a GSL-degrading glycosidase, as for example GBA. The degradative flux through GBA is considerable since its substrate GlcCer is generated in lysosomes from all glycosphingolipids (gangliosides, globosides and lactosylceramide). Despite the considerable *in utero* turnover of GlcCer, mice and humans without GBA develop more or less normally as fetus[24,25]. Only at birth the impairment of skin barrier function becomes fatal in the GBA-deficient collodion baby variant of GD. This skin defect is not attributed to lysosomal lipid storage, but rather an incorrect ratio of Cer and GlcCer in the extruded lamellar bodies forming the stratum corneum[26]. Upon autopsy marked GlcCer deposition is detected in several organs of the collodion baby, but organ function and development do not seem overtly impaired, at least until birth[27,28]. Another example in the same line are males with classic FD lacking any residual GLA protein. This 49 kDa lysosomal enzyme degrades the globoside Gb3 that is abundant in endothelial cells, blood cells, cardiomyocytes and podocytes[25]. Classic FD males with complete deficiency of GLA express only at juvenile age overt symptoms in skin, nociceptive neurons and eye[29]. Deposits of Gb3 in so-called zebra-bodies are already detected in fetal endothelial cells, but pathology of heart, kidney and brain only manifests in adult life[29]. Male FD mice lacking GLA show already in the first months of life prominent Gb3 storage in tissues, but this does not progress further[30,31]. Similar leveling of storage lipid deposition with age occurs in LIMP-2 deficient mice (Gaspar *et al.*, to be published; chapter 5 of this thesis). LIMP-2 essentially mediates the transport of newly synthesized GBA to lysosomes[32]. In the endoplasmic reticulum LIMP-2 binds GBA and the complex is sorted to late endosomes/lysosomes where the acid pH causes dissociation[32,33]. Although most cell types and tissues of LIMP-2 deficient mice are severely deficient in GBA, this is accompanied by marginally increased GlcCer. Moreover, the storage of GlcCer in LIMP-2 mice does not increase with age (Gaspar *et al.,* to be published; chapter 5 of this thesis). The non-linear increase in GSL storage with age in GLA- and LIMP-2 deficient mice is puzzling since it cannot be explained by a simple feedback regulation. No prominent reduction of GSL biosynthesis in response to defective lysosomal degradation is reported. This contrasts sharply to the tight regulation of cellular cholesterol by sensing of sterol concentrations in membranes and the transcriptional and post-translational regulation of biosynthetic and other modifying enzymes[34]. The sensing of lysosomal dysfunction and subsequent responses have been elucidated by Ballabio and colleagues. It has become clear that lysosomal stress by indigestible macromolecules leads to translocation of the transcription factor TFEB to the nucleus. There, it increases transcription of genes encoding protein constituents of lysosomes and

autophagosomes[35]. Although increased *de novo* synthesis of entire lysosomes may contribute to reducing lysosomal lipid storage at short term, the amelioration is ultimately limited by the total cellular volume that can be occupied by lysosomes.

Thus, the paradox presented by the glycosphingolipidoses is the initial rapid accumulation of GSL followed by a far less progressive phase. An explanation for this riddle would be induction of cellular adaptations in metabolism of GSLs in response to increasing storage in lysosomes (figure 2). Such adaptive responses would fit with the observed leveling of GSL storage with age. At present, the nature of the postulated adaptations is largely unknown.

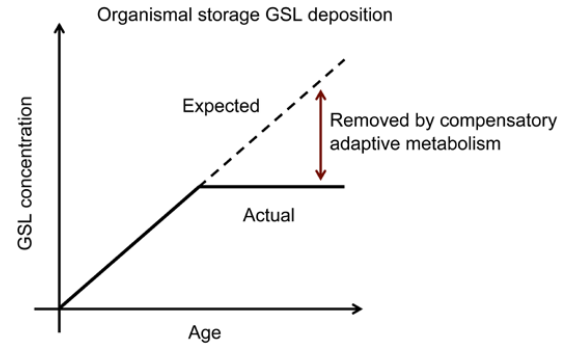


Figure 2. The paradox of glycosphingolipidoses. Theoretical argument for existence of adaptive metabolism for GSL clearance.

Adaptive rescue by lysosomal conversion of glycosphingolipids to glycosphingoid bases. Glycosphingoid bases corresponding to the primary accumulating GSLs in cells and tissues are markedly increased in plasma of patients suffering from glycosphingolipidoses, (figure 3A). Examples are the 300-fold elevated GlcSph in plasma of GD patients. More modestly, GlcSph is also increased in plasma of GBA-deficient patients suffering from Action Myoclonus Renal failure Syndrome (AMRF) resulting from mutations in the *SCARB2* gene encoding LIMP-2[36–38]. Likewise, lysoGb3 is 200-fold increased in plasma of classic FD patients[39,40]. Furthermore, galactosylsphingosine is increased in Krabbe disease (KD) patients deficient in galactocerebrosidase degrading GalCer[41,42]. Examination of glycosphingoid bases in mouse models with deficiencies in glucocerebrosidase, α-galactosidase A and galactocerebrosidase recapitulated these findings. In plasma and tissues of all mice, the storage of the primary GSL substrate is accompanied by marked increases in corresponding glycosphingoid bases[43].

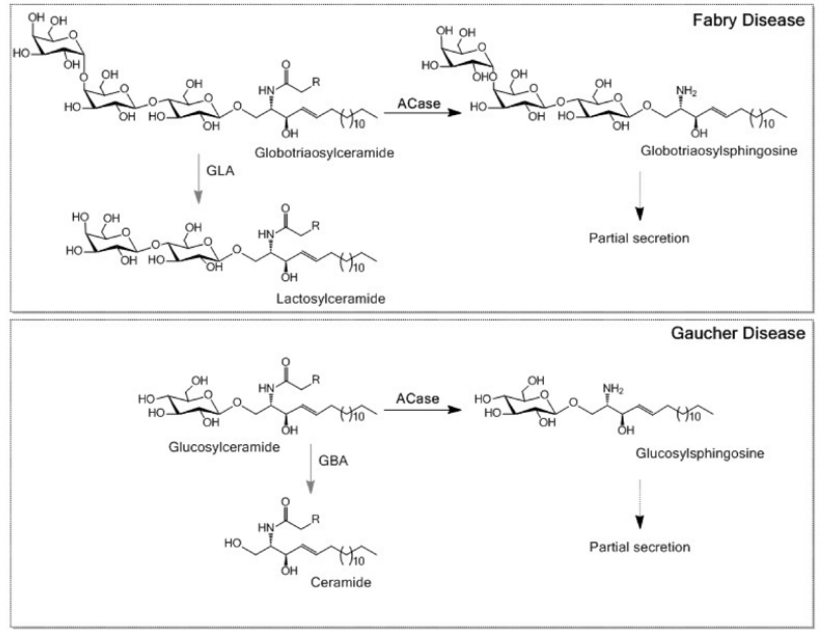
The question to be addressed next is "how do the excessive amounts of glycosphingoid bases arise?". A first clue was offered by the work of Yamaguchi *et al*. demonstrating that pharmacological inhibition of GBA in fibroblasts results in formation of GlcSph, but not in cells lacking the lysosomal enzyme AC[44]. The putative role of lysosomal AC in formation of glycosphingoid bases was further investigated[45]. The prominent generation of GlcSph upon

inhibition of lysosomal GBA was found to be abolished by genetic loss of AC as well as its pharmacological inhibition[45]. Feeding of cells with $^{13}C_5$-isotope encoded GlcCer (containing the isotope label in the sphingosine moiety) led to prominent formation of $^{13}C_5$- GlcSph when GBA was inhibited in cells. The same observation was made for classic FD fibroblasts: inhibition of AC also abolished the generation of lysoGb3 by these cells[45]. Thus, AC is able to convert accumulating GlcCer and Gb3 in lysosomes to the corresponding glycosphingoid bases GlcSph and lysoGb3. The amphiphilic nature of these glycosphingoid bases allows export from lysosomes and even cells[45]. GlcSph and lysoGb3 are water soluble contrary to their corresponding GSLs. The glycosphingoid bases are not associated with plasma lipoproteins like GlcCer and Gb3 are. In urine, GlcSph and lysoGb3 are not in the proximal tube cell sediment, but recovered in the cell-free supernatant. The concentrations of glycosphingoid bases in plasma and urine correlate with each other, both in FD and GD patients. Most of the urinary GlcSph in GD patients and lysoGb3 in FD patients is more hydroxylated and possibly methylated in their C18-sphingoid base as compared to the major species in plasma[37,46] (figure 3B). These modifications in the bases seem to be directly introduced in the kidney since they are lacking for urinary GlcCer and Gb3. Possibly, local monooxygenase activity of CYPs contributes to the modifications. Another structural heterogeneity observed for plasma as well as urinary GlcCer, GlcSph, Gb3 and lysoGb3 is the presence of an additional double bond in the sphingosine moiety. These 4*E*,14*Z*-dienes usually constitute around 10 % of the total glycosphingolipids and glycosphingoid bases.

Formation of glycosphingoid bases, a blessing or a curse?

The conversion of accumulating GSLs into corresponding glycosphingoid bases during deficiency of GBA or GLA explains, at least in part, the non-linear increase of GSL in time in GD and FD (figure 2). The lysosomal de-acylation by AC provides the organism a way to eliminate indigestible GSL as water-soluble glycosphingoid bases from cells and even the body by excretion via bile and urine (figure 3A). This solution mimics the body's handling of excessive non-digestible cholesterol, being secreted as such in bile or as water-soluble metabolite like bile acid. LIMP2-deficient mice illustrate how de-acylation of non-digestible GlcCer allows to efficiently prevent lysosomal storage in most cells. This is exemplified by the liver of LIMP2-deficient mice with near complete absence of GBA. Microscopic examination shows no storage deposits and biochemical analysis reveals only a very modest increase in GlcCer. Concomitantly, hepatic GlcSph is 6.7-fold increased and biliary GlcSph is 4.5-fold elevated (Gaspar *et al.,* unpublished; chapter 5). The normal liver function of LIMP2-deficient mice is likely achieved by the ability to avoid formation of lipid-laden lysosomes in hepatocytes.

A)



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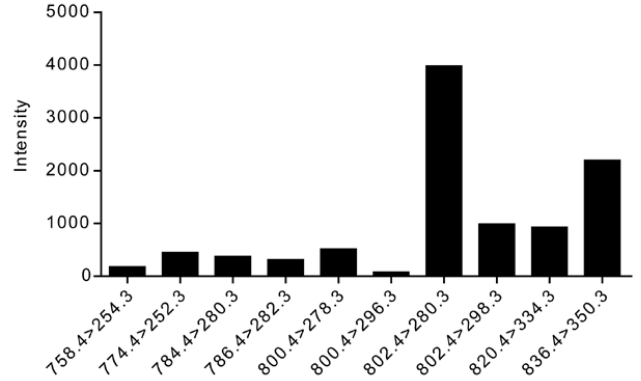

Figure 3. AC-mediated deacylation of GlcCer and Gb3. A) Deacylation by AC of GlcCer and Gb3 to GlcSph and lysoGb3, respectively. **B)** Isoforms of lysoGb3 found in the urine of 2 classical Fabry patients.

There is a prize to pay for the adaptive de-acylation of non-digestible GSL in lysosomes: it exposes the body to chronic high levels of glycosphingoid bases (figure 4). This does not seem to be without long term health risk. Glycosphingoid bases are biologically active and may exert negative effects (for detailed reviews see references[25,47–50]). Briefly, the production of galactosylsphingosine in brain of KD patients contributes to the devastating neuropathology[50].

GlcSph, chronically elevated in GD patients and to lesser extent AMRF patients, is found to be toxic at high concentrations. For example, GlcSph experimentally promotes lysis of red blood cells, impairs cell fission during cytokinesis, damages specific neurons, hampers growth, impairs bone formation by osteoblasts, and promotes chronic inflammation via activation of phospholipase A2[47,48]. It is appealing to speculate that GlcSph contributes to the occurrence of hemolysis, multinucleated macrophages, neuropathology, growth retardation, bone deterioration and chronic low grade inflammation in GD patients. Gammopathies are common in GD patients[51]. Studies by Cox and co-workers firstly demonstrated a correlation between lymphoma and plasma GlcSph levels in mice with inducible GBA knock-down in the white blood cell lineage[52,53]. Recently, Nair and colleagues reported that excessive GlcSph in GD patients may act as auto-antigen driving B-cell proliferation. Thus, GlcSph would directly promote development of multiple myeloma, a blood cell cancer occurring with increased incidence in GD patients[54]. LysoGb3, excessively generated in classic FD patients, is also considered to be toxic[55,56]. It has recently been proposed that lysoGb3 through sensitization of nociceptive neurons plays a direct role in the intense pain experienced by FD patients[57]. Indeed, plasma lysoGb3 in FD patients has been earlier found to correlate with pain[56]. LysoGb3 is also considered as culprit in renal disease in FD patients by causing podocyte damage and fibrosis in the kidney[58]. A correlation of plasma (or urinary) lysoGb3 with renal complications has however not yet been documented. Finally, there is experimental evidence that lysoGb3 promotes proliferation of smooth muscle cells *in vitro* which might explain the characteristic increase in intima media thickness in vasculature of FD patients[39,55]. In theory, GlcSph and lysoGb3 might also be harmful as structural mimic of sphingosine-1-phosphate (S1P), interfering with processes governed by this sphingoid base and its receptors[59]. Minor abnormalities in S1P have been reported for FD patients[60,61]. Of note, the putative toxic effects of GlcSph in GD1 and lysoGb3 in classic FD occur relatively late in patients' lives, implying that associated pathologies require prolonged exposure to high concentrations of glycosphingoid bases. In conclusion, the potential toxicity of glycosphingoid bases urgently warrants further examination.

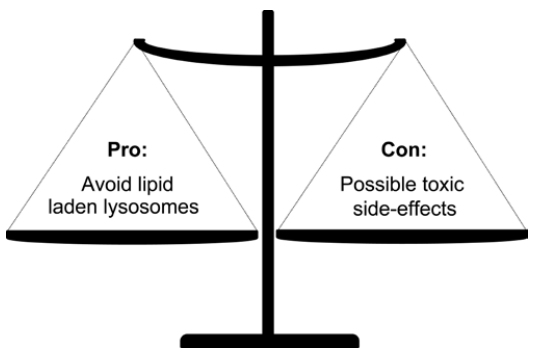

Figure 4. Pro and con of GSL base formation. Pro: avoidance cellular dysfunction by accumulation of dysfunctional lipid laden lysosomes; con: possible toxic side effects.

Employing glycosphingoid base abnormalities for diagnostic purposes and disease monitoring.

The abnormal high concentrations of glycosphingoid bases in blood and urine of patients with a glycosphingolipidosis can be used to biochemically confirm diagnosis and to demonstrate onset of pathological GSL accumulation[25,36,39–41,62,63] (addendum of this thesis). The recent development of very sensitive methods for the quantification of glycosphingoid bases in complex biological samples has been a major step forward[40] (addendum of this thesis). Glycosphingoid bases like GlcSph and lysoGb3 can be in parallel accurately quantified by LC-MS/MS with use of $^{13}C_5$ isotope-encoded natural sphingoid bases as internal standards (figure 5)[37,40,64,65]. With these advanced LC-MS/MS methods, average 300-fold increases in plasma GlcSph in symptomatic GD patients and 200-fold increased lysoGb3 in plasma of males with classic FD were detected (figure 5D)[37,64]. Nowadays, GD and FD patients are identified in screening programs based on detection of gene abnormalities or reduced enzyme activity in dried blood spots. Diagnosis based on these tests is sometimes ambiguous, for example in the case of mutations with unknown consequence or marked residual enzymatic activity. Demonstration of elevated GlcSph allows sensitive and reliable confirmation of diagnosis for GD[37]. Likewise, markedly elevated plasma lysoGb3 confirms diagnosis of FD in males[64]. Glycosphingoid base abnormalities already occur in GD patients and male Fabry patients at very young age, prior to overt symptomatology. Marginal increases in plasma glycosphingoid bases should however be treated with caution since these might not be related to the presumed primary defect. For example, we noted that plasma samples of GD patients show besides more than 100 times increased GlcSph also a modest elevation in lysoGb3 level[45,66]. This is highly relevant since in recent years individuals with abnormalities of unknown significance in the *GLA* gene are increasingly regarded to be at risk for an atypical manifestation of FD[66,67]. Contrary to classic FD patients, atypical patients express no characteristic acroparesthesias and corneal clouding early in life, but only develop one of the isolated late onset symptoms such as unexplained stroke, cardiomyopathy or renal disease. The relative high frequency of such symptoms in the general population makes it conceivable that simply by mere chance an individual with a *GLA* polymorphism develops such a common symptom. It should be avoided that in these cases a faulty diagnosis of FD is made. An incorrect FD diagnosis in a male has serious consequences since all daughters are labelled as obligate carriers that potentially develop disease and require preventive, extremely costly, therapeutic intervention by enzyme replacement therapy. To avoid faulty diagnoses a threshold value of 1.3 pmol/mL plasma lysoGb3 was earlier proposed to distinguish true atypical FD patients from individuals with alpha-galactosidase A abnormalities without significance[68]. This threshold was based on data from 10 non-matched controls. Plasma specimens from individuals with unexplained stroke, cardiomyopathy or renal disease in the presence of normal GLA were not analysed. The strict use of the proposed threshold 1.3 pmol/mL cannot be recommended since plasma lysoGb3 levels in nearly every GD patient examined exceeds this. It is conceivable that diverse causes for lysosomal stress, including

chronic exposure to lysomotropic drugs, may cause non-specific modest elevations of glycosphingoid bases in plasma (up to a few pmol/mL).

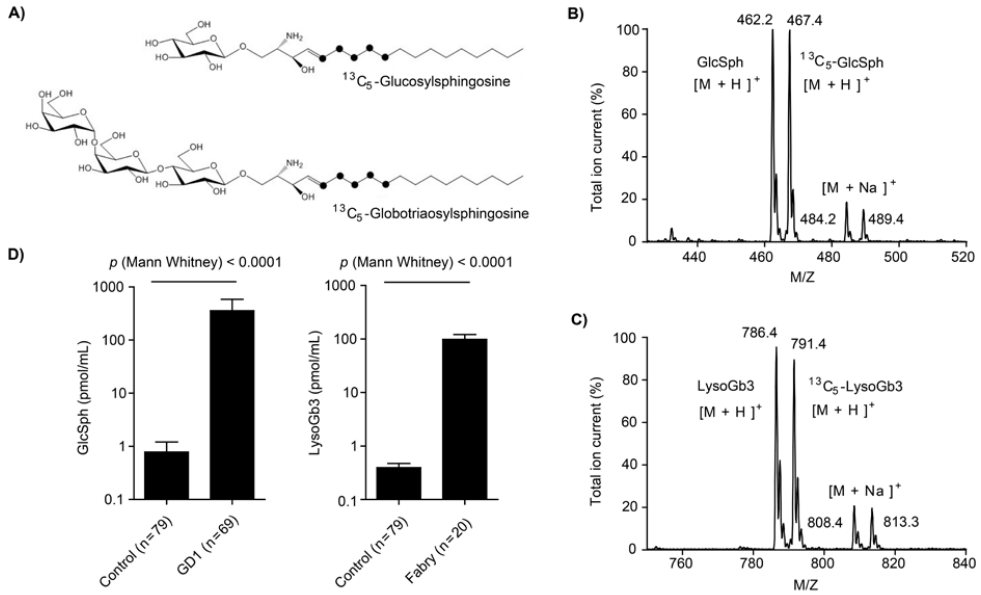

Figure 5. LC-MS/MS quantification of glycosphingoid bases. A) $^{13}C_5$-encoded isotope standards of GlcSph and lysoGb3. **B)** M/z ratio for analyte and internal standard for GlcSph. **C)** M/z ratio for analyte and internal standard for lysoGb3. **D)** GlcSph levels in GD1 patients (n=69) and lysoGb3 in classical FD patients (n=20).

Another application of measurement of glycosphingoid bases is found in monitoring disease manifestation and progression in GD1 patients and classic FD patients[36,69–73]. The origin of circulating glycosphingoid bases is by virtue unknown. Consequently, plasma glycosphingoid bases do not reflect a particular symptom. Regular measurements of plasma glycosphingoid bases in GD and FD patients receiving costly enzyme replacement therapy should nevertheless be advocated since this renders objective information on general efficacy of the intervention. A lack of response in plasma GlcSph of GD patients, or plasma lysoGb3 of FD patients, receiving therapy strongly suggests that the treatment is ineffective. Striking is the noted fast relapse in plasma GlcSph in GD patients following ERT interruption or major dose reductions as well as relapses in plasma lysoGb3 in classic FD males following the formation of neutralizing antibodies against the therapeutic enzyme[69,72]. The measurement of glycosphingoid bases is also of great value to examine the efficacy of experimental therapeutic intervention in animal models. An illustration for this forms the work of Dahl and colleagues on gene therapy in mice with an inducible loss GBA activity in white blood cells[74]. These mice, and similar animals generated by Mistry and colleagues[75] provide valuable

information on the relationship of plasma GlcSph and GlcCer-laden macrophages (Gaucher cells). The induction of Gaucher cells in both mouse models was found to be associated with a marked increase of plasma GlcSph, indicating that these cells are a major source of the circulating glycosphingoid base. Another strong indication for this are the earlier noted proportional changes in plasma GlcSph and chitotriosidase, a validated biomarker for Gaucher cells in most GD patients receiving ERT[36].

Out of the lysosome box: metabolism of GlcCer by the cytosol-faced enzyme GBA2.

The present paradigm on GSL degradation includes its restriction to lysosomes. An exception to this forms GlcCer, the only GSL synthesized and present in the cytosolic leaflet of membranes. Two decades ago the existence of a non-lysosomal glucosylceramidase, presently named GBA2, was discovered[76]. The enzyme differs from GBA in sensitivity for inhibitors and ability to degrade artificial β-xyloside substrates[76]. The strong membrane association of GBA2, and its intrinsic lability upon dissociation from membranes with detergents, hampered purification. Cloning of GBA2 cDNA, independently reported by Yildiz *et al.* and colleagues and Boot *et al.*[77,78], shed the first light on the protein's structural features. GBA2 proves to be a non-glycosylated 927-amino acid protein (figure 6A) that is synthesized in the cytosol and subsequently strongly binds to membranes[78]. Literature reports on its subcellular localization are conflicting, ranging from endosomes to the endoplasmic reticulum[78,79]. GBA2 lacks a true transmembrane domain and most likely the catalytic pocket is inserted in the cytosolic leaflet of membranes, consistent with the early observation that the enzyme preferentially uses substrate while embedded in the membrane[76]. At present, no 3D-structure of GBA2 is available. Very recently a crystal structure was published for the slightly homologous TxGH116 β-glucosidase from Thermoanaerobacterium xylanolyticum, revealing a N-terminal domain, primarily formed by a two-sheet β-sandwich and a C-terminal (α/α)6 solenoid domain[80]. The C-terminal domain contains the residues that were proposed as catalytic nucleophile and general acid/base in the archaeal β-glucosidase from Sulfolobus solfataricus and human GBA2[81]. The residues binding the glucose in the −1 subsite are highly conserved between TxGH116 β-glucosidase and human GBA2[80]. The enzyme is a retaining β-glucosidase using double displacement in catalysis with glutamate 527 as nucleophile and aspartate 677 as acid/base[81]. Hydrophobic iminosugars like AMP-deoxynojirimycin (AMP-DNM, IC_{50} of 1 nM) ((figure 6B) and N-butyl-deoxynojirimycin (Zavesca; IC_{50} of 250 nM)) are potent inhibitors of GBA2[82,83]. A survey of genomes shows that GBA2 is an ancient and evolutionarily conserved protein[84]. GD patients treated for more than a decade with *Zavesca* at concentrations that inhibit GBA2 activity generally tolerate the drug well. On the other hand, there are several reports of patients with defects in the GBA2 gene developing spastic paraplegia and cerebellar ataxia[85–90]. Their disease has an early onset and involves muscle weakness and spasticity in upper and lower limbs, cognitive impairment ataxia, axonal neuropathy and cerebellar and cerebral atrophy. Likewise, knock-down of GBA2 with antisense morpholino oligonucleotides in zebrafish led to abnormal motor behavior and axonal shortening/branching of motor

neurons[91]. However, GBA2 knock-down in mice does not cause any neuropathology[77] (figure 6C). GBA2-deficiency in male mice reduces spermatocyte fertility due to a defect in acrosome formation in early post-meiotic germ cells and causes malformation of the sperm head[92]. These defects are attributed to disorganization of cytoskeletal structures[93]. GBA2 mice also show impaired liver regeneration associated with cytokine- and growth factor-mediated signaling pathways[94].

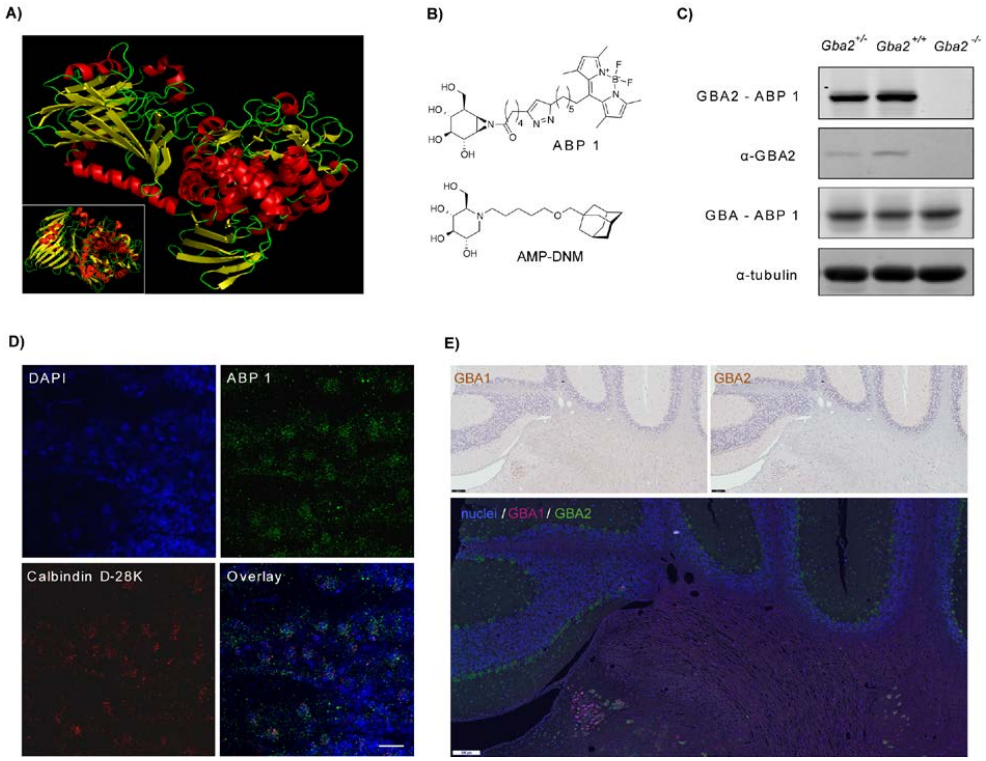

Figure 6. GBA2 *in silico*, *in vitro* and *in vivo*. A) Homology model of GBA2 secondary structure. **B)** Chemical structure of nanomolar GBA2 inhibitor AMP-DNM and activity-based probe (ABP 1) targeted against GBA and GBA2. **C)** Labeling of GBA2 and GBA by ABP 1 and immunoblotting of GBA2 and tubulin in brain homogenates of mice heterozygous, wt and knock-out for *Gba2*. Scale bar = 20 µm. **D)** *In situ* visualization of GBA2 labeled *in vivo* following i.c.v. injection of ABP 1. **E)** Immunostaining of GBA and GBA2 in the cerebellum of wt mouse. Scale bar = 100 µm.

The discovery of GBA2 in the nineties soon led to the speculation that this enzyme might be over-active in GD and that excessive degradation of GlcCer by GBA2 may exert toxic effects contributing to GD symptomatology[95]. Mistry and colleagues tested this hypothesis many years later by crossing in GBA2 deficiency in mice with inducible GBA deficiency. Indeed, the

absence of GBA2 significantly rescued the clinical phenotype of the GD mice[96]. Cells from GD patients have been reported to show higher levels of GBA2 protein as well as increased *in vitro* enzymatic activity when compared to corresponding control cells[97,98]. Recently, an opposite down-regulation of GBA2 activity in GD was reported and ascribed to its inhibition by sphingosine[99]. No explanation is at present available for the conflicting findings. In Niemann-Pick type C, the levels of GBA are significantly reduced in cells and tissues and GlcCer is elevated[100–102]. To test the hypothesis that GBA2 activity during GBA deficiency is harmful, the effect of genetic loss of *Gba2* in *Npc1*^{nih} mice developing major motor neuron loss was examined. NPC mice with concomitant GBA2 deficiency lived longer and developed loss of motor coordination at later age (figure 7C)[98]. The survival of Purkinje cells, motor neurons with a relatively high GBA2 content, is prolonged in GBA2-deficient NPC mice (figure 6D-E)[98]. The beneficial effect of reducing GBA2 in NPC mice was recapitulated by daily administration of 1 mg AMP-DNM per kilo to each animal, a dose sufficient to inhibit GBA2 in the brain of mice (figure 7B). This investigation and that by Mistry and colleagues[96], provide evidence that GBA2 activity during GBA deficiency has harmful effects. The molecular mechanism for this remains currently elusive. Excessive generation of Cer from GlcCer by GBA2 in the cytosolic membrane leaflet might be detrimental (figure 7A) in view of the presumed pro-apoptotic role of cytosolic Cer and its stimulation of inflammation through activation of PLA2 resulting in increased prostaglandin E2[95].

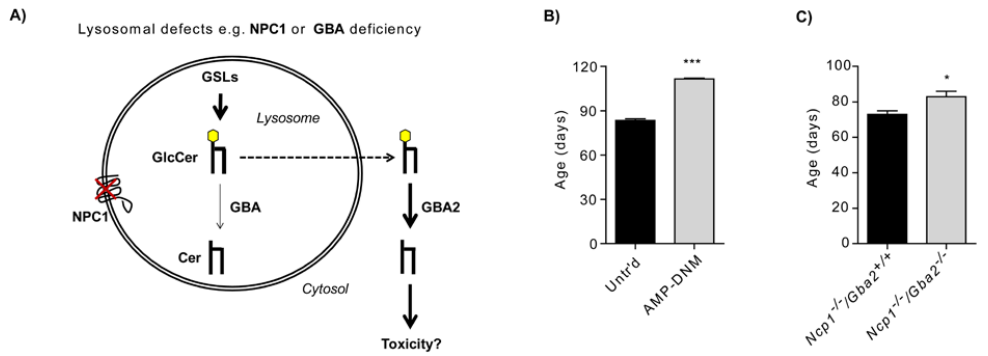

Figure 7. Detrimental role of excessive GBA2 activity during GBA deficiency. A) Scheme of postulated excessive compensatory activity of GBA2 during GBA deficiency. **B)** Mean survival of *Npc1*^{nih} mice treated with one mg per kilogram per day of AMP-DNM in the diet and untreated control. **C)** Mean survival of *Npc1*-deficient mice wt or knock-out for *Gba2*.

GBA2, a β-glucosidase generating GlcChol by transglucosylation.

The catalytic mechanism of the retaining β-glucosidases GBA and GBA2[103], coined 'double-displacement' by its discoverer Koshland Jr., deserves closer inspection (figure 8A). The catalytic pocket of these enzymes employs two adjacent carboxylic acid residues, spaced ~5.5 Å apart, with one acting as catalytic nucleophile and the other as acid/base residue. The

deprotonated carboxylate of the nucleophile attacks the substrate's anomeric C1 carbon, while the carboxylic acid side-chain of the acid/base donates a proton to the inter-glycosidic oxygen. The aglycone is next expelled while concurrently a glycosyl-enzyme intermediate is formed, with an inversed configuration at the anomeric center. To deglycosylate the enzyme, the now deprotonated side-chain of the acid/base abstracts a proton from an incoming water molecule, forming a nucleophilic hydroxyl that attacks the anomeric center (C1) of the glycosyl-enzyme adduct and causes release of the sugar with overall retention of configuration (figure 8A). Of interest, several retaining glycosidases can also transglycosylate, i.e. transfer the sugar from substrate to an acceptor other than a free hydroxyl. A thorough historical account of transglycosylation by glycosidases is provided by the review of Hehre[104]. For example, acceptors in the transglycosylation by chitinases are sugars[105]. Glew and co-workers showed that GBA can catalyze the transfer of the glucose from 4-methylumbelliferyl-β-glucoside to retinol and other alcohols[106]. Akiyama and colleagues demonstrated that *in vitro* GBA generates 25-NBD-cholesterol-glucoside from GlcCer and artificial 25-NBD-cholesterol[107]. Marques and colleagues recapitulated their finding with natural cholesterol as acceptor[102]. Artificial β-glucosides like 4-methylumbelliferyl-β-glucoside as well as natural GlcCer were suitable sugar donors to generate glucosyl-β-D-cholesterol or 1-O-cholesteryl-β-D-glucopyranoside (GlcChol) (figure 8B). It was next discovered that also the enzyme GBA2 can generate GlcChol *in vitro* through transglucosylation, again using GlcCer as donor. Expectedly, GlcChol also proved to be an excellent substrate for *in vitro* hydrolysis by GBA and GBA2.

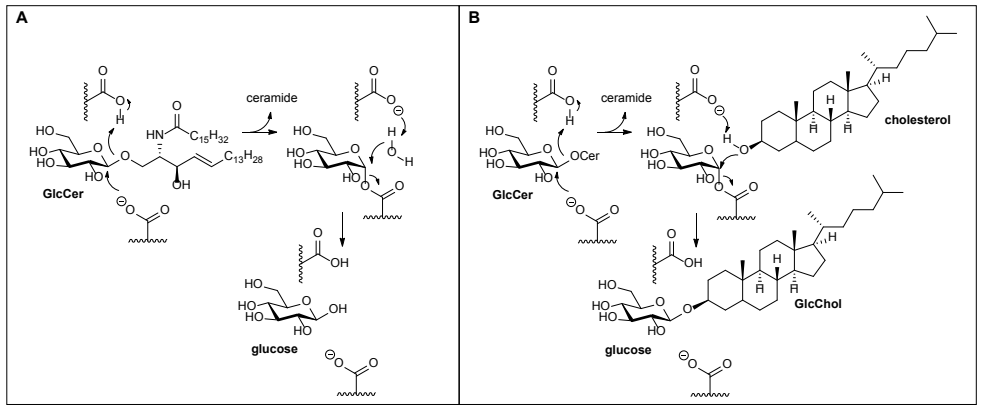

Figure 8. Transglucosylation. A) Hydrolysis of GlcCer by a β-glucosidase yielding free glucose and ceramide. **B)** Transglycosylation of cholesterol catalyzed by a β-glucosidase using GlcCer as donor of the glucose moiety and leading to the formation of GlcChol. Shown in both schemes are the catalytic residues of the β-glucosidase: acid-base (top) and nucleophile (bottom). See also ref[102].

The existence of sterol-glucosides in plant and fungal species is well documented[108], but not for mammals. Murofushi and co-workers proposed the presence of GlcChol in cultured human fibroblasts and gastric mucosa, but solid analytical proof for this was not provided[109,110]. To establish physiological relevance of GlcChol, its natural occurrence in mammalian cells and tissues was investigated. For this, a sensitive quantitative detection of GlcChol by LC-MS/MS using $^{13}C_5$-isotope labeled GlcChol as internal standard was developed. GlcChol was subsequently detected in human plasma and cultured cells. GlcChol was found to be present in all examined tissues of mice[102]. The highest concentration was observed for sciatic nerve[102]. The relative high amounts of GlcChol in the thymus, several nanomoles per gram of wet weight, are of interest in view of noted abnormalities in NKT and B-cells in GBA-deficient GD patients[111–113]. It has been proposed by Mistry and colleagues that elevated GlcCer or GlcSph via binding to CD1 may be causing this[111], but a role for GlcChol should not be excluded in this respect.

The biosynthesis and degradation of GlcChol in cells have been elucidated[102,114]. The enzyme GCS (EC2.4.1.80) forms GlcCer by transfer of glucose from UDP-glucose to Cer[115]. However, GCS does not synthesize GlcChol as firstly demonstrated by Akiyama and colleagues[114]. Next, Marques and co-workers studied GD mice, GBA-deficient LIMP-2 KO mice and GBA2-deficient mice to determine whether GBA or GBA2 is responsible for formation of GlcChol through transglucosylation[102]. Mice deficient in GBA showed modestly elevated GlcChol in several tissues. GBA2-deficient animals presented a very marked reduction in GlcChol in tissues, suggesting that *in vivo* GBA2 largely forms GlcChol and GBA degrades it. Consistent with this interpretation is the observed increase in plasma GlcChol in symptomatic GD patients.

Intrinsically, the local concentrations of donors (GlcCer and GlcChol) and acceptors (ceramide and cholesterol) determine the transglucosylation equilibrium of a retaining β-glucosidase. This is nicely illustrated by the finding that high lysosomal cholesterol concentrations drive GBA to generate GlcChol instead of degrading it[102]. The induction of lysosomal cholesterol accumulation in cells with U18666A causes a rapid increase in GlcChol, which is abolished by selective inactivation of GBA[102]. Consistently, in liver of NPC mice GlcChol is 25-fold elevated[102]. Pharmacological inhibition of GCS leads to reduction of GlcChol in cultured cells and plasma of mice and GD patients[102]. Apparently, availability of GlcCer is essential for formation of GlcChol through transglucosylation. GBA2 is well positioned in the cytosolic membrane leaflet containing GlcCer and cholesterol to generate GlcChol.

The physiological function of GlcChol is at present unclear. GlcChol is far more water soluble than cholesterol and intrinsically more suited for non-vesicular transport between compartments. Tentatively, water soluble GlcChol formed by transglucosylation at one cellular site could be transported to another site. A reverse reaction at the destination site could reconverted back cholesterol without any need for ATP. During pathological conditions such as GD, secondary abnormalities in GlcChol likely occur due to the imbalance in GBA and GBA2 activities. Future research will need to address whether such abnormalities in GlcChol, or in other glucosylated metabolites, contribute to particular GD symptoms.

Conclusion and Outlook

As reviewed, there is compelling evidence for the occurrence of adaptive metabolism in glycosphingolipidoses like GD and FD. Firstly, there is solid proof for formation of glycosphingoid bases in response to intralysosomal accumulation of GSLs. Secondly, there is strong evidence pointing to cytosolic metabolism of GlcCer by GBA2 that may generate GlcChol as side product and this pathway seems increased during GBA deficiency. Other metabolic adaptations in these diseases might still have to be discovered. A potential alternative compensatory reaction comes to mind, i.e. direct enzymatic removal of the entire glycan of GSLs. Such enzymes actually do exist in nature and are named endoglycoceramidases (EGC)[116,117]. At present several EGCs, with little mutual sequence homology, have been identified in mollusk, leech, earthworm and several pathogenic cestode parasites[118]. A search for the existence of such enzymes in mammals seems warranted, particularly since a comparable enzymatic activity has been reported by Basu *et al.* for rats and specific tumor cell lines[119–122]. The recent availability of activity-based probes recognizing various retaining β-glucosidases might assist the discovery of an elusive EGC in mammals. Briefly, cyclophellitol-epoxides and cyclophellitol-aziridines with a particular sugar configuration react with high specificity with corresponding retaining glycosidases through irreversible linkage to the catalytic nucleophilic residue. Cyclophellitol-epoxides tagged at C6 with a fluorophore react specifically with GBA, while cyclophellitol-aziridines tagged at C1 with a fluorophore react with several β-glucosidases[123,124]. Bacterial EGCase has already been found to react well with a β-glucopyranosyl-configured cyclophellitol-aziridine tagged with either a fluorophore or biotin[125]. The biotin-tagged ABP might be employed in a search for the elusive mammalian endoglycoceramidase and facilitate its purification and subsequent identification by proteomics.

The recent recognition of toxic effects of excessive lysoGb3 in FD and excessive GlcSph in GD deserves attention and warrants further research. Could novel drugs be envisioned to ameliorate these pathological effects by specifically reducing the glycosphingoid bases? Unfortunately, inhibition of AC, the enzyme responsible for generation of glycosphingoid bases, seems wise not since this will cause impaired lysosomal degradation of ceramide mimicking Farber disease, a severe neurological disorder[126]. The realization that excessive GBA2 during GBA deficiency is detrimental for motor neurons should be pursued regarding therapy. Further investigations on the therapeutic value of available brain-permeable combined inhibitors of GBA2 and GCS[127] are appealing for the juvenile neuronopathic variant of GD (type 3), in particular given the positive effect of the well tolerated inhibitor AMP-DNM in NPC mice[98,126] and similar positive findings made earlier by other investigators with Sandhoff mice[128], another glycosphingolipidosis.

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