Glycosphingolipids and the central regulation of metabolism
Herrera Moro Chao, D.

Citation for published version (APA):
Herrera Moro Chao, D. (2017). Glycosphingolipids and the central regulation of metabolism: Sugar analogues as research tools

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
General introduction
General introduction

Historical preface. Already in the late 19th century the basis for modern neurochemistry was laid by the seminal investigations of Johannes Ludwig Thudichum on the chemical composition of the human brain. The combined work was published as monograph titled A Treatise on the Chemical Constitution of Brain in 1884 [1]. Following successful studies on the chemical composition of gall-stones, bile and urine, Thudichum focused attention to the brain and soon discovered the presence of numerous distinct lipid molecules with hitherto unknown structure. Among these, he identified lipids built upon sphingoid base backbones for which he coined the name “sphingosin” in “commemoration of the many enigmas which it has presented to the inquirer” (ref 1, p 149). At about the same time, January 1882, Philippe C.E. Gaucher described in his doctorate thesis at the university of Paris, titled De l’epithelioma primitif de la rate, hypertrophie idiopathique de la rate sans leucemie, a 32-year old female patient whose spleen and liver were enlarged and showing abnormal deposits in reticulo-endothelial cells (tissue macrophages) [2]. Within a few decades it was recognized that the patient represented a distinct inheritable disease entity that was subsequently named Gaucher disease. In 1924 chemical analysis of Gaucher disease spleen by the German physician Lieb led to the discovery of gross accumulation of a glycosphingolipid, a cerebroside as originally coined by Thudichum [3]. In his paper Cerebroside speicherung bei Splenomegalie Typus Gaucher Lieb erroneously assumed that the storage material was galactosylceramide, a glycosphingolipid later shown to accumulate in the inherited disorder Krabbe disease caused by deficiency of the β-galactosidase named galactocerebrosidase [4-6]. The correct structure of the storage lipid in Gaucher disease as glucosylceramide (glucocerebroside) was described by Aghion in his PhD dissertation titled La Maladie de Gaucher dans l’Enfance [7]. Only in 1965, Brady et al. and Patrick independently linked Gaucher disease to deficient activity of acid β-glucosidase, named glucocerebrosidase [8-10].

This thesis builds on the fundamental discoveries made in the late 19th century. It contains investigations on glycosphingolipids and their metabolizing enzymes in the brain using state-of-the art techniques. Synthetic sugar analogues were employed to visualize glycosidases in their real life context and to modulate metabolism of glucosylceramide. Iminosugar-based pharmacological modulation of glucosylceramide metabolism was earlier found to result in pleiotropic beneficial effects in obese rodents, including improved satiety, a function well known to be regulated by the brain [11-13]. Further elucidation of the suggested link between glycosphingolipids, central regulation of food intake and bodily metabolism, was a major research objective of this thesis study.

Glycosphingolipids. Glycosphingolipids (GSLs) are structural components of membranes, particularly present in the outer leaflet of the plasma membrane bilayer. In the brain specific glycosphingolipids are also abundant in myelin, the fatty sheaths surrounding as electrically insulating layer the axons of neuronal cells. Schwann cells myelinate the peripheral nervous system and oligodendrocytes axons of the central nervous system. The generic structure of glycosphingolipids exists of a ceramide lipid moiety with attached to the C1-hydroxyl a monosaccharide (glucose or galactose) to which further sugars may be added [14]. In the case of galactosylceramide further sulfation at the 3-hydroxyl occurs in the Golgi apparatus resulting in the lipid named sulfatide, both major components of myelin. Through van der Waals interactions with cholesterol molecules GSLs form transient semi-ordered domains in plasma membranes. Specific proteins preferably reside
in these so-called ‘lipid rafts’ where they mediate signaling events [15-17]. GSLs travel through various subcellular compartments during their life cycle. The ceramide backbone is initially synthesized at the endoplasmic reticulum (ER) in a multi-step process. Serine and palmitoyl-CoA are conjugated by the enzyme serine palmitoyltransferase (SPT) to generate a keto-sphinganine [18]. This is next transformed by a reductase to sphinganine that subsequently can be N-acylated by any of a set of ceramide synthases (CerS 1-6) with different acyl-CoA length preference [19]. The formed dihydroceramides are next converted to ceramides by dihydroceramide desaturase (DES) [20, 21]. A fraction of the newly formed Cer reaches the lumen of the ER where it may be converted to galactosylceramide (GalCer) by galactosylceramide synthase using UDP-galactose as sugar donor [22]. Alternatively, the protein CERT transports Cer molecules to the cytosolic leaflet of membranes of the cis-Golgi apparatus [23]. Here, glucosylceramide (GlcCer) is generated by the enzyme glucosylceramide synthase (GCS), which transfers a glucose from UDP-glucose [24, 25]. A major part of the formed GlcCer is next translocated to the luminal leaflet of the Golgi membrane via an unknown mechanism [25]. Inside the Golgi apparatus, GlcCer is next modified by stepwise addition of further sugars catalyzed by glycosyltransferases, yielding various types of complex GSLs such as gangliosides and globosides [14, 26]. Sulfation of specific lipids, most prominently galactosylceramide, by sulfotransferases may occur, contributing to the structural diversity of GSLs [26, 27]. After their processing in the Golgi apparatus, GSLs reach the outer leaflet of the plasma membrane where they fulfil various functions in interactions with the outside world. A unique situation forms the myelin sheath where extended plasma membrane of Schwann cells or oligodendrocytes is wrapped in multiple layers around the axons of the nerve cells [28, 29]. GSLs are internalized from the cell surface via endocytosis, becoming part of multi-vesicular bodies within late endosomes. Subsequently, their degradation inside lysosomes occurs. Exogenous GSLs, for example components of phagocytosed cell debris and senescent cells or endocytosed lipoproteins, likewise undergo degradation after reaching lysosomes. This degradation implies step-wise removal of terminal sugar moieties from GSLs by sequential action of glycosidases, assisted by specific accessory proteins (GM2 activator protein and saposins A-D) [30]. In the process, ultimately Cer is formed, either from GalCer by galactocerebrosidase (GALC) or GlcCer by glucocerebrosidase (GBA). Cer is split by the lysosomal acid ceramidase (AC) to free fatty acid and sphingosine. Following export to the cytosol, sphingosine may be re-used by CerS enzymes in the salvage pathway to generate again Cer molecules [31]. Alternatively, sphingosine is metabolized by sphingosine kinases (SK1 and SK2) to sphingosine-1-phosphate (S1P). Catalyzed by S1P lysase (SPL), this is degraded to phosphatidylethanolamine and 2-trans-hexadecenal [32, 33].
It has recently become apparent that during pathological conditions (see also section below) GSLs may be largely alternatively degraded in lysosomes [35, 36]. When accumulating in lysosomes, GSLs such as GlcCer, GalCer and Gb3 (globotriaosylsphingosine) may be converted by AC to their corresponding sphingoid bases glucosylsphingosine, galactosylsphingosine and lysoGb3 (globotriaosylsphingosine), respectively [37-40]. These glycosphingoid bases are amphiphilic and can leave lysosomes and subsequently cells. The metabolism of glucosylceramide has a unique aspect. Being the sole GSL generated in the cytosol it is uniquely subject to metabolism there. The membrane-associated enzyme GBA2, a β-glucosidase that is non-homologous to GBA [41-43], is able to remove the glucose from GlcCer. Besides catalyzing hydrolysis of GlcCer it may transfer the glucose from the lipid to a suitable acceptor like cholesterol [44]. This transglucosylation generates glucosyl-cholesterol (GlcChol), a water-soluble sterol with yet unknown function.

**Glycosidases.** Lysosomes contain a set of glycosidases allowing fragmentation of nearly all glycoconjugates. Glycosidases are often classified as retaining or inverting according to the stereochemical outcome of the hydrolysis reaction [45]. Glycosidases are furthermore grouped as exo or endo enzymes, dependent on cleavage at the end or in the middle of a saccharide chain. Lysosomal exo-glycosidases essentially contribute to the degradation of GSLs. The features of GALC and GBA as well as the cytosolic glucosylceramidase GBA2 warrant special description because they are subject of detailed investigation in this thesis.

**Galactocerebrosidase: galactosylceramide hydrolase.** GALC is a retaining β-galactosidase, encoded by the GALC gene at locus 14q3. Being synthesized as 669 amino acid polypeptide at ER-associated ribosomes with a regular N-terminal signal sequence of 26 amino acids, it is co-translational translocated to the lumen of the ER where the signal peptide is removed and potentially 6 glycans are attached [46]. The glycans of GALC acquire mannose-6-phosphate (M6P) recognition signals in the Golgi apparatus allowing selective binding to M6P receptors and subsequent sorting to lysosomes. The precursor GALC is approximately 80-85 kDa and converted in lysosomes to the active form con-
sisting of two subunits of 50-52 kDa and 30 kDa. GALC as retaining glycosidase employs the double displacement mechanism [47]. GALC shows optimal enzyme activity at pH 4.6. The catalytic activity of GALC towards galactosylceramide is improved by interaction with the small accessory proteins saposin A and saposin C generated by proteolytic processing of the precursor prosaposin [48]. The 3D-structure of GALC has been resolved by X-ray crystallography, revealing a TIM-barrel structure, commonly encountered in glycosidases, containing the catalytic residues [46, 47]. The domain architecture of GALC is unique including a lectin-domain not yet encountered in other hydrolases. Disease-causing mutations have been detected throughout the protein [45, 49].

**Glucocerebrosidase: glucosylceramide hydrolase.** GBA is a retaining β-glucosidase, encoded by the GBA gene at locus q21 of chromosome 1 [50]. It is synthesized as 497 amino acid polypeptide at ER- associated ribosomes with a regular N-terminal signal sequence allowing co-translational translocation to the lumen of the ER [51]. Inside the ER, the signal peptide is removed and glycans are attached to the amino acids N19, N59, N146 and N270 of the nascent GBA, an essential modification for correct folding of the protein [52, 53]. Next, within one hour of their synthesis, folded GBA molecules bind to the triple helical structure in the apical region of integral membrane protein LIMP-2 (lysosomes integral membrane protein 2, encoded by the Scarb2 gene) containing trafficking information in its cytoplasmic tail [54, 55]. Incorrectly folded GBA molecules fail to associate with LIMP-2 and are subsequently removed from the ER to be degraded in proteasomes [56]. Contrary to most other lysosomal hydrolases, including GALC, GBA does not acquire M6P moieties and is not subject to M6P receptor mediated delivery to lysosomes [57]. Instead, bound to LIMP-2 GBA traverses the Golgi apparatus where most of its N-glycans are modified to complex type- structures [54]. The journey of newly formed GBA from the ER to lysosomes is in some cultured cells surprisingly slow, taking several hours [58]. The survival of GBA in lysosomes is relatively short (t1/2 ~24-36 hours), at least in cultured cells. The intralysosomal proteolytic breakdown of GBA is mediated by cysteine proteins as suggested by its inhibition by leupeptin and E64 [59, 60]. After reaching acid late endosomes/lysosomes, GBA dissociates from LIMP-2 [61]. In the acid lysosomes the enzyme GBA meets optimal conditions for catalytic activity promoted by saposin C, an 80 amino acid protein generated from prosaposin [62]. Saposin C stimulates enzymatic activity of GBA towards GlcCer, presumably by facilitating entry of lipid substrate in the catalytic pocket [30]. GBA utilizes during catalysis the double-displacement mechanism like most other retaining glucosidases. The key catalytic residues in GBA are the nucleophile glutamate 340 and acid/base glutamate 325 [63]. As reaction intermediate the glucose of GlcCer substrate becomes covalently linked to E340 and is released by subsequent attack of a nucleophilic water molecule assisted by E235. The 3D-structure of GBA has been solved by X-ray diffraction crystal analysis, indicating a (β/α)8 TIM barrel catalytic core domain III, a three-strand antiparallel β-sheet flanked by a loop and a perpendicular strand (domain I) and an Ig-like fold formed by two β-sheets (domain II) [64, 65].

**Cytosolic glucosylceramidase GBA2.** Studies on the degradation of C6-NBD-GlcCer in cultured cells with normal and deficient GBA rendered the first indication for the existence of a second glucosylceramidase in cells that was coined non-lysosomal glucosylceramidase or GBA2 [41]. The enzyme is strongly associated to membranes and its activity is lost upon extraction from membrane with detergents, hampering purification and further characterization for almost a decade. Independently, Yildiz and co-workers and Boot and colleagues cloned the gene encoding GBA2 (locus 1p13) [42, 43]. It is now apparent that
GBA2 is synthesized as cytosolic protein that binds via an unknown mechanism tightly to membranes. Very likely its catalytic pocket is imbedded in the cytosolic leaflet of the membrane as suggested by the noted kinetics of substrate degradation [41]. GBA2 is a retaining β-glucosidase with prominent transglucosylase capacity. It appears largely responsible for the (reversible) formation of GlcChol from GlcCer in cells and tissues [44]. Given the membrane embedded position of its pocket the enzyme seems ideally positioned for the transfer reaction of glucose among membrane lipids. Different subcellular localization of GBA2 has been reported, ranging from endosomes to endoplasmic reticulum [41, 66]. It is not clear whether this can be attributed to the distinct cell types analyzed or differences in methodologies used. At present no 3D-structure of GBA2 has been obtained. There exists partial homology with a β-glucosidase from *Sulfolobus solfataricus* [67], which assisted the identification of the catalytic residues, being E527 as nucleophile and D677 as acid/base [63]. Very recently a crystal structure was published for the weakly homologous TxGH116 β-glucosidase from *Thermoanaerobacterium xylo- lanolyticum*, revealing a N-terminal domain with a two-sheet β-sandwich, and a C-terminal (α/α)6 solenoid domain. The proposed as the catalytic nucleophile and general acid/base in the archaeal β-glucosidase from *Sulfolobus solfataricus* and human GBA2 are in the C-terminal domain of TxGH116 β-glucosidase. The residues binding the glucose in the −1 subsite are conserved between TxGH116 β-glucosidase and human GBA2 [68]. Wachten and co-workers demonstrated that in GBA2 knockout-mice cytoskeletal dynamics are abnormal, most likely due to a more ordered lipid organization in the plasma membrane [69]. In dermal fibroblasts, actin polymerization is augmented, leading to a higher number of lamellipodia and filopodia and enlarged microtubules. Male germ and Sertoli cells from GBA2 knockout-mice show comparable cytoskeletal abnormalities. F-actin structures in the ectoplasmic specialization were found to be abnormal as well as microtubules in the sperm manchette. These disturbances might explain the defect in spermatogenesis earlier noted in GBA2-deficient mice [42, 70-73].

**Sugar analogues as mechanism-based probes for glycosidases.** Cyclophellitol and conduritol B-epoxide (CBE) irreversibly inhibit GBA by forming a permanent conjugate with the nucleophile E340 of GBA [74]. The irreversible inhibition of GBA by cyclophellitol was exploited for the design of Activity based probes (ABPs) for the enzyme [75, 76]. Initially, β-glucopyranosyl-configured cyclophellitol-epoxides modified at C6 (glucopyranose numbering) with a fluorescent BODIPY were designed and shown to covalently bind, with high specificity in mechanism-based manner, to the catalytic nucleophile residue E340 of GBA [75]. *In situ* labeling of active GBA in intact cells with the ABPs is feasible, visualizing the intralysosomal location of the enzyme. Reductions in active GBA molecules can be detected after the exposure of cultured GD fibroblasts to ABP and subsequent analysis of ABP-labeled protein by gel electrophoresis and quantitative fluorescence scanning [75].

Intravenous infusion of mice with the ABPs results in specific labeling of GBA in various tissues, except brain and eye [76]. This is likely explained by the active removal of the BODIPY-containing compounds by P-glycoproteins in the endothelial cells of brain capillaries. In chapter 1 of this thesis it is shown how intracerebroventricular administration of the ABPs to mice allows the visualization of active GBA in brain with high spatial resolution [77].

The successful design of fluorescent β-glucopyranosyl-configured cyclophellitol-epoxides directed to GBA directed ABPs was next extended to β-galactopyrano-
syl-configured structures. Their reactivity with GALT, the lysosomal β-galactosylceramidase deficient in Krabbe disease, was studied. In chapter 2 of this thesis the positive outcome of these investigations is described.

Next, a broad spectrum ABP was generated by the design of β-glucopyranosyl-configured cyclophellitol-aziridines with a fluorophore modification [76]. In these probes the fluorophore is positioned more closely to the position of the aglycon in substrates of β-glucosidases. Therefore, these ABPs covalently label a broad class of human β-glucosidases, including GBA, GBA2, GBA3 and lactase-phloridzin hydrolase [76]. In chapter 1 of this thesis investigations on the cellular localization of both GBA and GBA2 in the brain of mice are described.

The ABPs should find application in the diagnosis of lysosomal storage diseases as well in fundamental research. Of note, the ABPs can be applied cross species given the conserved catalytic pockets of glycosidases. They can be also equipped with biotin instead of fluorophores, allowing convenient purification by means of streptavidin-based pull down and subsequent identification of proteins by proteomics [78, 79]. This procedure should also allow identification of interacting proteins with the ABP-targeted enzyme. The GBA ABPs can be further optimized to monitor the precise localization of enzyme in life cells for which super-resolution microscopy or correlative light and electron microscopy can be used. For this cyclophellitol derivatives can be linked with a norbonene at C8 (cyclophellitol numbering) to generate a mechanism-based inhibitor projected to be (due to the bulk at this position) selective for GBA. Pulse labeling of tissue culture and washing away unbound probe can be followed by treatment with fluorogenic tetrazine. Only after inverse-electron demand Diels Alder the dye becomes fluorescent, thus limiting background labeling [80].

Figure 2. Concept of labelling targeted enzymes by ABPs in vitro and in vivo with different read-out possibilities. Based on Kallemeijn, et al [81].

Glycosphingolipidoses. Deficiency of lysosomal glycosidases and their accessory proteins involved in GSL degradation has been linked to an inherited metabolic disease with characteristic clinical presentation and characterized by lysosomal accumulation of some GSL [82-84]. This group of inherited lysosomal storage disorders is collectively named the glycosphingolipidoses. The clinical picture of glycosphingolipidoses varies considerably. In many cases, the defects in lysosomal catabolism often manifest as central nervous system (CNS) pathology. Lysosomes are essential in the recycling of macro-molecules and membranes delivered by autophagy and endocytosis. In view of this it is not surprising that defects in the compartment are particularly detrimental for long-lived neuronal cells. Indeed, a role for lysosomes has been proposed in pathogenesis of sever-
al neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer disease, and Parkinsonism [85-89]. However, Fabry disease (lysosomal α-galactosidase A deficiency) does not involve CNS pathology at all. Remarkable are the differences in the sequence and nature of neuropathology among the inherited glycosphingolipidoses [34]. Two glycosphingolipidoses warrant a detailed introduction: Gaucher disease (GBA deficiency) and Krabbe disease (GALC deficiency).

**Gaucher disease: impaired lysosomal GlcCer degradation.** Defects in GBA cause intralysosomal accumulation of GlcCer in characteristic tubular structures [90, 91]. Inherited mutations in the GBA gene constitute the molecular basis for the relatively common lysosomal storage disorder named Gaucher disease (GD). At present more than 200 mutations in the GBA gene are linked with GD [70]. Next to truncations and splicing defects, several hundred amino acid substitutions in GBA have been shown to cause GD [92]. The position of amino acid substitutions in the protein, in the catalytic or another domain, proves to poorly predict the clinical severity of GD patients [40]. Some substitutions in the folding domain that are positioned far away from the catalytic pocket have nevertheless major negative consequences. For instance, the substitution L444P in GBA causes faulty folding of most enzyme molecules in the ER and subsequent proteasome mediated degradation [94]. Homozygosity for L444P GBA nearly always leads to a severe neuronopathic course of GD, albeit with great individual variability in onset and progression [90]. Premature degradation may also occur with mutations in the catalytic domain. In fact, quite many of the documented mutations in GBA lead to defective folding and reduced transport to lysosomes [94]. An exception is the N370S GBA substitution, the prevalent GBA mutation among Caucasians GD patients. The amino acid substitution is in a loop close to the catalytic pocket and found to affect pH optimum and kinetic parameters such as affinity for substrate [56, 95, 96]. The intralysosomal stability of N370S GBA is markedly reduced [59, 96-99]. GBA genotype – GD phenotype correlation is surprisingly poor [100], illustrated most explicit by the occurrence of phenotypically discordant monozygotic twins [101, 102]. The clinical presentation of GD is remarkably heterogeneous, ranging from fatal skin defects to an almost asymptomatic course of disease [90]. The most common phenotype among Caucasian GD patients is referred to as type 1 or non-neuronopathic GD. Prominent in these patients is the ongoing storage of GlcCer in lysosomes of tissue macrophages, transforming into the characteristic enlarged lipid-laden Gaucher cells [103, 104]. Progressive accumulation of these alternatively activated lipid-laden macrophages in tissues of GD patients is supposed to underlie the development of symptoms like splenomegaly and hepatomegaly [90]. Massive accumulation of Gaucher cells in the bone marrow is thought to contribute to hematological abnormalities, like anemia and the commonly encountered thrombocytopenia. Other, highly variable, signs and complications associated with GD are skeletal deterioration, neuropathology including oculomotor apraxia and peripheral neuropathy as well as polyclonal and monoclonal gammopathies [90]. GD patients developing lethal complications in the central nervous system are classified as type 2 GD (infantile onset) and type 3 GD (late infantile/juvenile onset). Complete deficiency of GBA activity results in pre-natal/neo-natal phenotypes characterized by lethally aberrant permeability of the skin (so-called collodion baby showing severe ichthyosis) [105-107]. Carriers of mutant GBA alleles that do not develop Gaucher cells and characteristic GD symptoms, intriguingly however have, like patients, a significantly increased risk for α-synucleinopathies such as Parkinsonism and Lewy-body dementia [108-110].
Relatively little is known about the pathophysiological mechanisms underlying the various symptoms manifesting in GD patients. It is doubtful that the presence of lipid-accumulating Gaucher cells in tissues explains the entire spectrum of symptoms and signs in GD patients. Gaucher cells are viable and secrete various proteins promoting the influx of further monocytes to disease loci, promoting ongoing inflammation and tissue remodeling [102, 103]. Splenomegaly is a very common sign of disease and associated with accumulation of Gaucher cells in the organ. The same holds for hepatomegaly. The correlation with the presence of Gaucher cells is less clear for other liver-related symptoms such as the frequent occurrence of gall-stones. Cirrhosis is rare and associated with a more severe disease course in general. Frequent in GD patients is polyclonal gammopathy, and quite often this evolves into monoclonal gammopathy [111]. Ultimately this can even lead to the development of multiple myeloma and amyloidosis [112-113]. Bone marrow infiltration by Gaucher cells is another regular sign in GD patients and likely contributes besides splenomegaly to the common thrombocytopenia and anemia, albeit the latter generally develops only in more severely affected patients [90]. The skeletal disease and bone remodelling in GD patients is heterogeneous and focal of nature [90]. It seems not to correlate well with other disease manifestations and circulating biomarkers of Gaucher cells [115]. Osteoporosis is often encountered in GD patients, potentially linked to impaired osteoblasts rather than increased osteoclast activity [115, 116]. Ichtyotic skin disease is observed only in those patients with virtual no GBA activity. The ratio of glucosylceramide to ceramide in the stratum corneum, determined by GBA, seems critical for correct barrier function of the skin [105-107]. Growth retardation and signs of insulin resistance point to hormone disturbances, again also occurring in milder affected type 1 GD patients [90, 117]. Cardiac valve calcification is a symptom specifically occurring in GD patients with D409H mutated GBA, suggesting a very specific, but still enigmatic, mechanism [90, 118-121].

Neuropathology in GD. Peripheral neuropathy has recently been recognized in GD patients with an otherwise relative mild disease course and with significant residual GBA activity [122]. Clinical symptoms related to the central nervous system such as epilepsy, apraxia and scoliosis are usually observed only in type 2 and 3 neuronopathic (nGD) patients with markedly reduced GBA activity, but on the other hand even GD carriers show an increased risk for Parkinsonism [108, 112]. The neuropathology in type 2 and 3 GD patients may comprise strabismus, saccade initiation failure, supranuclear gaze palsy, slow object tracking, hypertonia, rigidity, opisthotonus, bulbar palsy, seizures, ataxia, myoclonus, dementia, and mental retardation [90, 123-125]. Sub-categories have been proposed for the very heterogeneous juvenile nGD: type 3a with progressive dementia, ataxia, and myoclonus; type 3b with extensive visceral and bone involvement and CNS pathology limited to supranuclear gaze palsy (saccade initiation failure, with compensatory head thrusting); type 3c with corneal opacity, supranuclear gaze palsy, and cardiovascular calcification, combined with limited visceral disease. The remarkable clinical heterogeneity among GD patients has led to the proposal to consider the disorder rather as continuum of disease manifestations, variable among individual patients [123]. Experimental investigations trying to link abnormalities to specific neuropathology have been conducted in models of nGD generated in \((Gba\text{flox/flox};\text{nestin-Cre})\) mice lacking GBA in neuronal cells but not microglia [126-128]. These investigations have pointed out that activated microglia and neuroinflammation are associated with damaged brain areas. It is unclear whether inflammation occurs prior to neuron death or concomitantly, however in both scenarios anti-inflammatory drugs might be beneficial. Human nGD
brain presents a selective loss of neurons in cortical layers III and V [124]; likewise in nGD mice the cortical layer V is affected. In nGD mice, the motor and somatosensory cortex is more affected than the caudal visual cortex. Substantia nigra and red nucleus pathology occurs in nGD mice as seen in GD patients’ brain [124, 126]. The storage lipids GlcCer and glucosylsphingosine accumulate comparably in various brain regions, not correlating with local pathology [127]. Futerman and co-workers reached the conclusion that a certain level of neuronal GlcCer storage triggers neuropathology in affected brain areas, while other brain areas containing similar glucosylceramide levels remain unaltered, presumably due to intrinsic differences in neuronal properties or in the neuronal environment. An alternative nGD mouse model can be generated by treatment of mice with conduritol B-epoxide (CBE) [74, 129, 130]. CBE is an irreversible inhibitor of GBA, but not entirely specific given its simultaneous inactivation of glucuronidase and α-glucosidase. When administered at relative low dose (25-100 mg/kg), CBE is thought to preferentially inhibit GBA and generate a genuine GD model with comparable increases in GlcCer and glucosylsphingosine and brain pathology as observed in Gba<sup>flox/flox;nestin-Cre</sup> mice [130]. The investigations by Futerman and colleagues with CBE-treated animals have revealed the remarkable impact of the genetic background of mice. C57BL/6 mice poorly tolerate inhibitor treatment, resulting in death at day 20 at a dose of 100mg/kg, whereas other strains of mice survive this treatment much longer. The precise cause for this variability is not known, although it has been speculated modifier genes might underlie this. Members of the receptor-interacting protein kinase-3 (Ripk3) pathway (Rip1, Rip3 and Pkr) were found to be elevated in brain of CBE-treated mice, although to a lower extent compared to Gba<sup>flox/flox;nestin-Cre</sup> mice [131]. Of note, modulation of the Ripk3 pathway was found to markedly improve neurological and systemic disease in the nGD mice. Ripk3 deficiency increased survival and motor coordination of nGD mice and exerted beneficial effects on cerebral as well as hepatic injury [131]. Based on these findings, neuronal death in nGD is proposed to be due to necrosis mediated by Ripk3 and to be not caused by regular apoptotic mechanism [131]. Earlier it has been concluded that activated microglia is a key mediator in nGD pathology. It was hypothesized that once a critical threshold of GlcCer storage is reached in neuronal cells, a signal triggers microglia, which in turn releases inflammatory cytokines that amplify the inflammatory response, contributing to neuronal death [126]. More recently it has been reported that indeed a type 1 interferon response occurs in the brain of nGD mice [132]. Controversial views exist on the potential contribution of elevated glucosylsphingosine to the neuropathology of GD [125, 128]. The sphingoid base is consistently found to be markedly elevated in nGD brain of mouse and man; however it remains unclear whether local concentrations are sufficiently high to cause direct toxicity. Of note, no correlation has been noted between GBA activity levels, as measured in vitro, and neuropathology or accumulation of GlcCer and glucosylsphingosine. Consistent with this are findings for normal rat brain in which active GBA was visualized in situ with a specific fluorescent ABP [77]. Areas like the cortex and thalamus with prominent pathology in nGD mice do not present an exceptional content on active GBA molecules. It therefore seems that beyond the primary GBA other factors influence GD neuropathology. GBA2 might be one such modulating factor. The cerebellar cortex, specifically Purkinje neurons, shows prominent ABP labeling of GBA2, confirmed by antibody staining, specifically in. The prominent presence of GBA2 in cortex and thalamus coincides more closely with the areas involved in neuropathology in nGD mice as the brain distribution of GBA [77].

Another mechanism linked to the neurodegeneration observed in GD patients and oth-
LSD neurodegenerative disorders is the accumulation of insoluble oligomeric and fibrillar α-synuclein- positive inclusions known as Lewy bodies and Lewy neurites [108, 133]. Monomeric α-synuclein (14 kDa) is abundant in the brain, where it is thought to be involved in regulation of synaptic vesicles clustering and release of neurotransmitters through interactions with lipids in detergent-resistant membranes [134]. Similar to Parkinson’s disease and other α-synucleinopathies, GD patients present α-synuclein-positive Lewy bodies in the substantia nigra, cerebral cortex, hippocampus and a selective loss of dopamine neurons in the midbrain [133]. Different studies have presented complementary explanations for a reciprocal interaction between GBA and α-synuclein, overexpression of mutant GBA constructs in neuronal cells promoted accumulation of α-synuclein. Additionally, α-synuclein accumulation in the brainstem, hippocampus and substantia nigra was also observed in vivo in a mutant GBA mouse model or after chronic GBA inhibition by CBE administration [135, 136]. Biophysical studies have demonstrated that α-synuclein can also modulate GBA activity in a dose dependent manner [137], in vitro measurements have supported this view by showing that increased levels of α-synuclein can cause a reduction of wild-type GBA activity in neuronal cells [138], finally post-mortem analysis of Parkinson’s disease brains without GBA mutations, showed decreased GBA protein levels and activity in the cerebellum and substantia nigra [139]. It has been proposed that the dysfunction in the reciprocal balance between GBA and α-synuclein causes neurodegeneration [140] by impairing autophagy and lysosomal function [137]. Based on the vital interaction of GBA and α-synuclein, augmenting GBA activity directly in the substantia nigra or hippocampus [141, 142] or augmenting GBA expression by overexpression of basic helix-loop leucine zipper transcription factor EB (TFEB) [143] resulted in promising strategies to modulate α-synuclein aggregation and oligomerization in midbrain dopamine neurons.

**Pathological adaptations in GD.** Secondary abnormalities in GD patients stemming from adaptations to the primary lysosomal GlcCer accumulation most likely contribute to specific symptoms. One adaptation in GBA-deficient cells is the intralysosomal formation of GlcSph from accumulating GlcCer by AC. This seems pathogenic as such. Excessive GlcSph has been linked to B-cell lymphoma [144-146]. It has been recently reported that glucosylsphingosine in GD patients acts as auto-antigen driving B-cell proliferation and thus directly promotes the development of multiple myeloma, a blood cell cancer occurring with increased incidence in GD patients [144]. Of note, deacylation of accumulating storage GSL to corresponding glycosphingoid bases is not unique for GD and AMRF, but also occurs in Fabry disease and Krabbe disease [147, 148]. Again, toxicity of the generated glycosphingoid bases is considered: excessive galactosylsphingosine is thought to be neurotoxic in Krabbe disease patients (see below) [147] and excessive lysoGb3 is shown to be toxic for nociceptive peripheral neurons and podocytes in Fabry disease patients [149, 150]. It is presently unknown whether the chronically elevated glucosylsphingosine in GD patients causes other pathological manifestations besides multiple myeloma. Another compensation in GD is increased metabolism of GlcCer by the cytosolic β-glucosidase GBA2. Recently indications have been obtained that excessive activity of GBA2 might reduce motor coordination by Purkinje cell loss [151]. It might also lead to excessive formation of potential toxic metabolites such as ceramide and glucosylated compounds, e.g. GlcChol [44]. In addition, the deletion of the Gba2 gene in GD1 mice with a conditional GBA deficiency in white blood cells was found to rescue, fully or in part, osteopenia. Hepatomegaly, splenomegaly, cytopenia, - and hypercytokinemia [152].
Therapies for GD. Based on the pioneering studies by Brady and collaborators at the National Institutes of Health [153], an effective treatment for GD was firstly introduced, so-called enzyme replacement therapy (ERT) [154-155]. This treatment is based on two-weekly intravenous administration of glycan modified human recombinant GBA to supplement macrophages with enzyme. Initially GBA was isolated from human placenta and its N-glycans were enzymatically modified in vitro to expose terminal mannose residues favoring endocytotic uptake of enzyme via the mannose receptor, a lectin receptor present on tissue macrophages [156]. An effective ERT enzyme preparation was commercially developed by Genzyme (Boston, U.S.A.), involving large scale isolation of enzyme from placental extracts. The placental enzyme preparation has been replaced by a recombinant GBA produced in Chinese hamster ovary cells. Now several GBA preparations are in use for ERT of type 1 GD, all leading to reversal of organomegaly and hematological complications, stabilization of skeletal disease and markedly increased quality of life [157-161]. Clinical improvement is accompanied by corrections in plasma biomarkers of Gaucher cells and glucosylsphingosine [162]. The quantification of plasma chitotriosidase is widely applied to monitor disease progression and response to therapy. Corrections in plasma chitotriosidase of GD patients have been found to correlate with corrections in organomegaly and to be associated with improvements in hematological abnormalities. Moreover, the extent of correction in chitotriosidase correlates with the incidence of long term complications such as pulmonary hypertension, multiple myeloma and Parkinsonism [163]. ERT does not prevent neurological manifestations in more severely affected GD patients because the therapeutic enzyme fails to pass the blood-brain barrier [157]. An alternative treatment of type 1 GD is offered by so-called substrate reduction therapy (SRT) [164- 168]. Here, three times daily GD patients orally take a small compound inhibitor of GCS, the key enzyme in glucosylceramide and subsequent glycosphingolipid biosynthesis. Two drugs (Miglustat and Eliglustat) are approved for treatment of type 1 GD patients. Miglustat (N-butyl-deoxynojirimycin), already registered in 2001, is a relative weak and non-specific inhibitor of GCS. Lately the far more potent and specific GCS inhibitor Eliglustat (N-[(1R,2R)-1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(1-pyrrolidinyl)-2-propanyl]octanamide) has also been registered for SRT of type 1 GD patients [168-170]. The latter drug does not penetrate well the brain and is considered not suitable to treat neuronopathic variants of GD. The design of brain-permeable GCS inhibitors is actively pursued by pharmaceutical industry and academic researchers. Brain-permeable N-(5’-adamantane-1’-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM) [171] and idose variants thereof have been found to inhibit GCS with the same efficacy [172]. Based on these compounds, a new generation of deoxynojirimycin type GCS inhibitors with IC50 values in the very low nanomolar range has been recently developed [172].

There is active research on additional treatments for GD, particularly the neuronopathic variants for which there remains an unmet clinical need. Additional drugs considered are so-called chemical chaperones, small compounds interacting with the catalytic site of the enzyme that should chaperone folding of (mutant) GBA in the ER, resulting in increased transport of enzyme to the lysosome [173-176]. As many GD patients produce mutant forms of GBA that are impaired in folding and/or lysosomal stability, these might profit from such chaperone-based therapies. There is an explosive increase in reports on the design and synthesis of potential chemical chaperones for GBA. Recent reviews cover elegantly some of the classes of glycomimetics currently under investigation as chaperones [177, 178]. Many are reversible competitive, or mixed-type,
inhibitors of GBA. The most extensively studied small compound so far has been isofagomine (IFG) which was the subject of several pre-clinical studies as well as a clinical study that did however not meet the full expectation [179-188]. IFG is a potent competitive inhibitor of GBA with IC50 ~30 nM at pH 5.2 and 5 nM at pH 7.0. It only exerts a beneficial effect on GBA in a delicate concentration window that likely is difficult to reach simultaneously in various tissues. The effects of oral administration of Ambroxol, a weak mixed-type inhibitor of GBA, have meanwhile also been investigated in cells as well as a few type 1 GD patients [189-192]. Impressive reductions in spleen and liver volumes of Ambroxol-treated patients have been documented, associated with reductions of the GD biomarker chitotriosidase. Given the positive outcome of bone marrow transplantation in type 1 GD patients, genetic correction of hematopoietic stem cells has been, and still is, seriously considered as therapeutic avenue. Pioneering trials with retroviral vectors to introduce GBA cDNA in hematopoietic stem cells of GD patients did not give permanent correction of white blood cells [193], however in recent times encouraging data have been obtained with lentiviral gene therapy in type 1 GD mice [194]. Moreover, the exciting new possibilities for gene corrections using CRISPR-CAS technology may further promote a revival in research on gene therapy as therapeutic modality for GD [195].

**Krabbe disease: impaired lysosomal GalCer degradation.** Krabbe disease (KD) or globoid cell leukodystrophy is named after the Danish neurologist Knud Haraldsen Krabbe who firstly described in 1916 globoid cells (multinucleated macrophages) in the brain of a patient [4]. The disease affects the central and peripheral nervous system [5]. Affected at early stage is the cerebellum controlling movement and coordination of muscle activity. The disease progresses with loss of higher cerebral functions, while at later stage the loss of oligodendrocyte and myelin becomes prominent [196]. Cell deterioration or death activates an inflammatory response: recruited macrophages and activated microglia amplify the local production of cytotoxic molecules. The inflammatory response is followed by astrocytic gliosis. KD typically manifests at infancy, but milder phenotypes also occur [196]. The infantile phenotype usually starts in first 6 months of life and leads to death by age 2 years. Stiffness of the limbs, hyperirritability and hypersensitivity to the external environment and episodic fever of unknown origin are first signs. Psychomotor functions deteriorate rapidly accompanied by marked hypertonicity. At the final stage of the disease, the infants are blind and unresponsive. The more variable later-onset forms are divided into the late infantile (onset 6 months to 3 years), juvenile (onset 3–8 years) and adult forms. In the late infantile group, the first symptoms are hyperirritability, psychomotor regression, stiffness, ataxia and loss of vision. The disease progresses rapidly, causing death after 2 or 3 years of onset. The patients develop loss of vision, hemiparesis, ataxia, and psychomotor regression. Patients with adult onset show a slower rate of progression with a normal lifespan. Especially in the adult patients, genotype–phenotype relationship is poor [196, 197].

Not the primary substrate of GALC, the lipid GalCer, but rather its deacylated form galactosylsphingosine (psychosine) appears essential in pathogenesis of KD. GalCer does not prominently accumulate in KD in contrast to galactosylsphingosine [133]. Galactosylsphingosine has been shown to be highly cytotoxic [198, 199]. It interferes with various cellular processes by induction of pro-inflammatory cytokines such TNF-α and IL-6, inducible nitric oxide synthase (iNOS), phospholipase A2 (PLA2) activity and lyso-phosphatidylcholine generation. Moreover, it inhibits PI3K–Akt, PKC and AMPK activities [200]. It also interacts with the G protein-coupled orphan receptor TDAG8, thus inhibiting forskolin-induced cAMP formation [201]. Galactosylsphingosine further inhibits cy-
tokinesis, causing the presence of multi-nucleated globoid cells [200, 201]. At present, only haematopoietic stem cell transplantation, generally using umbilical cord blood, is available as KD treatment. The risky and evasive intervention has to be initiated before the onset of symptoms. Early diagnosis, differentiating infantile from late juvenile/adult onset forms, is therefore essential. Whilst reliable prognostication of disease course remains a challenge in some KD cases, pressure of patient societies has nevertheless led to the establishment of newborn screening programs [202]. The ethics of such screening are still debated given the large number of false-positives and often only partial success of transplantation [203]. Alternative treatments that are presently researched are gene therapy, substrate reduction therapy and chaperone therapy [204].

**GBA2 deficiency and clinical consequences.** Puzzling at the moment are the consequences of GBA2 deficiency. On the one hand, absence of GBA2 protein in mice has little detrimental effects except for the partial block in spermatocyte differentiation [42, 70-74], a defect that is even experimentally preventable by reducing glucosylceramide synthesis. Likewise GD patients treated with Zavesca, a nanomolar inhibitor of GBA2, generally tolerate the treatment well. On the other hand, there are now several recent reports of patients with defects in the GBA2 gene developing spastic paraplegia and cerebellar ataxia [205-211]. The disease has an early onset and involves muscle weakness and spasticity in upper and lower limbs, ataxia, axonal neuropathy, cognitive impairment, thin corpus callosum, and cerebellar and cerebral atrophy. Likewise in zebrafish knockdown of GBA2 causes neuropathology: antisense morpholino oligonucleotides against the zebrafish GBA2 orthologous gene caused axonal shortening/branching of motor neurons and abnormal motor behavior [208]. In sharp contrast, in GD mice with a conditional GBA knockout in white blood cells, concomitant GBA2 absence seems to ameliorate disease manifestation such as hepatosplenomegaly, cytopenia and osteopenia [152]. In the same line, Marques et al. reported how genetic loss of GBA2 as well as its pharmacological inhibition reduced loss of motor coordination in Niemann Pick type C mice and increased their life span [151]. No satisfactory explanation for this conundrum posed by the various data has yet been put forward.

**Glycosphingolipids and the cellular and body metabolism.** It has recently been recognized that lysosomes fulfil a key regulatory role in cellular metabolism. On the surface of lysosomes mTORC1 and AMPK are reciprocally activated, controlled by amino acid release from lysosomes, cellular energy status (AMP/ATP ratio) and glucose concentration (for excellent reviews on this topic see refs. 212-222). In an elegant way the sensing of nutrients at lysosomes is linked to the regulation of autophagy, de novo lysosome formation and lysosome exocytosis [212]. It has been demonstrated that the transcription factor TFEB regulates expression of genes coding proteins essential in autophagy and lysosomal biogenesis and that its translocation to the nucleus is controlled by mTORC1. In brief, activated mTORC1 on lysosomes, promoting anabolic processes in cells, actively retains TFEB from exerting its transcriptional action [212]. Vice versa, AMPK may replace mTORC1 on lysosomes to be locally activated in order to promote catabolic processes [222]. It is assumed that when lysosomes accumulate storage material the latter is favored, resulting in increased autophagy and lysosome biogenesis. Activation of AMPK is therefore considered favorable in the case of lysosomal storage disease [222].

AMPK activation has received considerable attention in the last decades as means to favorably change body metabolism in individuals that are overweight and show symptoms of the metabolic syndrome (see section below). For example, activated AMPK is
known to promote GLUT4 mediated glucose uptake, beta-oxidation of fatty acids, glycolysis and oxidative phosphorylation, whereas lipogenesis and sterol synthesis is reduced [222]. Of interest in this respect, are earlier findings on body metabolism in symptomatic type 1 GD patients. Resting energy expenditure was increased in symptomatic patients by approximately 24% and glucose production by approximately 30% and concomitantly insulin concentrations were slightly increased [223]. Only resting energy expenditure was noted to be reduced by enzyme replacement therapy [224]. GD patients show elevated levels of pro-inflammatory cytokines in combination with hypermetabolism [117, 225]. Follow-up investigations revealed that GD patients show signs of insulin resistance in combination with low serum adiponectin levels [226]. Gangliosides, in particular the ganglioside GM3, are markedly elevated in plasma and spleen of GD patients [227]. This finding led to the hypothesis that excessive ganglioside might impair insulin sensitivity in GD patients. The same hypothesis was earlier formulated by Hakamori and colleagues based on their finding of impaired responsiveness to insulin in tumor cells with high levels of gangliosides [228]. Next, Tagami et al. demonstrated that addition of GM3 to cultured adipocytes reduces glucose uptake by reducing phosphorylation of the IR and its down-stream substrate IRS-1 [229]. Later, Inokuchi and coworkers demonstrated that exposure of cultured adipocytes to TNFα increases the ganglioside GM3 [230] that directly interacts with K9444 of the IR and thus impairs signaling and GLUT4 translocation to the plasma membrane [230, 231]. Studies in rodents strengthened further the hypothesis by the demonstration that GM3 synthase knockout (GM3S−/−) mice are protected against high fat diet induced insulin resistance [232]. At the same time, Summers and co-workers put forward a causative role for excessive ceramide in the development of insulin resistance supported by mechanistic data (for reviews see refs. 233-239). Since ganglioside formation is closely linked to ceramide availability different views developed on the relative importance of both lipid species in the etiology of insulin resistance. However, a consensus is now reached in which excessive ceramide is thought to cause insulin insensitivity in muscle, whereas excessive ganglioside is the major culprit in adipocytes [233]. Studies applying quantitative lipidomics revealed inverse relationships between ceramide and glucosylceramide levels and insulin sensitivity in both rodents and humans [233]; the same holds for plasma GM3 [240]. In addition, strong relationships have been identified between inflammatory events, ceramide biosynthesis, and insulin resistance. Direct roles for adipokines in the modulation of cellular ceramide levels have been documented [233, 238]. Of note, leptin and adiponectin are found to reduce ceramide and upstream gangliosides whereas TNFα increase these sphingolipids [233].

**Iminosugars: Modulators of glycosphingolipid metabolism.** Given the growing evidence for a negative role of excessive gangliosides in insulin responsiveness, the effects of inhibitors of glucosylceramide synthase (GCS) have been extensively studied in overweight rodents showing signs of metabolic syndrome. Two categories of GCS inhibitors are presently known: N-substituted deoxynojirimycin iminosugars and ceramide analogues. Platt and Butters firstly recognized the ability of N-butyl-deoxynojirimycin iminosugars and ceramide analogues. Platt and Butters firstly recognized the ability of N-butyl-deoxynojirimycin to reduce glycosphingolipid biosynthesis via inhibition of GCS [241]. Meanwhile superior inhibitors of the iminosugar class were developed, amongst which N-(5’- adamantane-1’-yl-methoxy)-penty1-1-deoxynojirimycin (AMP-DNM) with a hundred fold greater potency and an improved bio-availability including penetrance in the brain [242, 243]. The ceramide analogue PDMP, a long time known inhibitor of GCS, was further modified to increase potency and specificity of inhibition, ultimately rendering Eliglustat (GENZ-123346) and
close analogues that do not enter the brain [244-246]. Very recently further improved and brain-permeable GCS inhibitors of the same class have been developed [247]. The iminosugar AMP-DNM and the structurally unrelated Genz-123346, a close analogue of Eliglustat, were tested in insulin resistant and diabetic mice and rats [248-251]. In obese, insulin resistant ob/ob mice lacking leptin, treatment with AMP-DNM was found to reduce blood glucose levels, improve glucose uptake from the circulation and increase suppression of endogenous glucose production by insulin. AMP-DNM also was noted to improve the insulin-stimulated IR phosphorylation in the liver and to reduce hepatic fat accumulation. Treatment with AMP-DNM of high fat diet-induced insulin-resistant mice and diabetic ZDF rats, AMP-DNM reduced fasting and non-fasting plasma glucose, corrected hyperinsulinemia, ameliorated glucose tolerance and reduced glycated hemoglobin levels. Similar beneficial effects were observed for Genz-123346 treatment [249]. In ob/ob mice and ZDF rats, AMP-DNM treatment corrected hepatic and muscle glucosylceramide and ganglioside content without affecting ceramide concentrations [248, 249]. GSL lowering was found to protect ZDF rats against loss of pancreatic beta-cells [249].

Figure 3. Chemical structures of the different iminosugars mentioned in the text.

It was noted that the oral treatment with AMP-DNM, albeit a poorer GCS inhibitor than GENZ-123346 as also reflected by less reduction of tissue gangliosides, improved better glucose homeostasis, suggesting an additional action of AMP-DNM next to GSL lowering. Miglitol (Glyset; N-2-hydroxyethyl-deoxynojirimycin) is a registered drug for treatment of type 2 diabetes through inhibition of sucrase-isomaltase (SI), resulting in buffering of complex carbohydrate assimilation [252, 253]. AMP-DNM is also a potent inhibitor of SI [254]. An L-idose-analogue of AMP-DNM was synthesized (L-ido-AMP-DNM) that was found to inhibit GCS on a par with AMP-DNM, but not SI [254]. Oral AMP-DNM treatment of obese rodents led to a superior improvement of glucose homeostasis as compared to equimolar L-ido-AMP-DNM. Next, it was demonstrated that oral AMP-DNM, but not L-ido-AMP-DNM, buffered the uptake of glucose from sucrose. Combined these findings suggest that AMP-DNM exerts a dual beneficial effect on glucose homeostasis, through sensitizing tissues to insulin and through buffering the assimilation of food carbohydrates by reducing activity of intestinal glycosidases [254].

Given the key role of inflamed adipose tissue in the metabolic syndrome, the effects of oral AMP-DNM on this tissue were also examined [255]. Drug treatment prevented formation of crown-like structure and reduced macrophage content of adipose tissue of obese mice. Inflammation of adipose tissue was markedly reduced by AMP-DNM as indicated by analysis of gene expression. Insulin responsiveness of adipose tissue, and isolated adipocytes thereof, was improved leading to reduced lipogenesis. Adipsin and adiponectin synthesis was normalized and the latter adipokine was present in plasma at higher concentration as beneficial high molecular weight form [255]. The observed anti-inflammatory action of AMP-DNM was also observed in liver. Strong suppression of inflammation by the compound was earlier noted in a mouse model of chemical-induced colitis [256]. The molecular mechanism by which AMP-DNM reduces tissue inflammation is at present not known. The effect seems not to depend only on reduction of glycosphingolipid biosynthesis since it is not observed to the same extent with GENZ-123346 and it occurs relatively acutely.
The outcome of oral GCS inhibitor treatment on rodent liver was investigated in quite some detail. Oral treatment of normal C57BL/6J mice for 5 weeks with AMP-DNM reduced plasma triglycerides and cholesterol by 35%, while biliary lipid secretion and fecal cholesterol excretion doubled. Expression of Cyp7A1, converting cholesterol to 7-alpha-hydroxycholesterol (the rate limiting step in bile acid synthesis) was up-regulated [257]. Exposure of the hepatoma cell line HepG2 to high dose AMP-DNM led to up-regulated expression of genes regulated by sterol regulatory element-binding protein (SREBP) 1 or 2. Increased cholesterol production confirmed the induction of SREBP target genes, but cholesterol content of the cells did surprisingly not concomitantly increase [258]. Nonalcoholic fatty liver disease (NAFLD) is associated with obesity as well as insulin resistance and type 2 diabetes. Hyperinsulinemia is believed to contribute to the development of fatty liver. AMP-DNM treatment of ob/ob mice improved hepatic insulin signaling, corrected blood glucose and insulin concentrations [259]. The hepatic expression of SREBP1c target genes involved in fatty acid synthesis normalized in the animals. AMP-DNM treatment decreased liver to body weight ratio and it reversed hepatic steatosis, including fat as well as inflammatory markers [260]. Inhibition of GSL synthesis in ob/ob mice with GENZ-123346 was also found to markedly reduce the development of hepatic steatosis [260]. As with AMP-DNM, GENZ-123346 treatment decreased expression of several genes involved in gluconeogenesis, lipogenesis and inflammation. Likewise, lowering GSL synthesis in diet-induced obese mice prevented steatosis and partially reversed pre-existing steatosis [260]. The effect of GSL synthesis inhibition on nonalcoholic steatohepatitis (NASH) was assessed in low-density lipoprotein receptor (LDLR) -/- mice on a western-type diet for 12 weeks to induce liver disease. Next, for 6 weeks the animals were fed with a diet with or without AMP-DNM. Drug treated mice presented less steatosis, inflammation and fibrosis in their livers. Induction of fatty acid beta-oxidation was detected, as well as a correction of plasma lipids [261].

Atherosclerosis is another clinical complication common in individuals with metabolic syndrome. The accumulation of sphingolipids (GSLs and sphingomyelin) has been reported for atherosclerotic lesions in animal models and humans. The effect of AMP-DNM on the development of atherosclerosis was therefore studied in two mouse models [262]. APOE*3 Leiden mice and LDLR -/- mice on a high-cholesterol diet were treated for 18 weeks with AMP-DNM. The treatment prevented hyperlipidemia. Moreover it led to a less atherogenic lipid profile and a marked reduction in atherosclerotic lesions in both models. At the highest dose of AMP-DNM, no lesions were even detectable. This effect of AMP-DNM was accompanied a decrease in liver cholesterol in combination with an increased excretion via bile and feces [262]. A follow-up investigation showed that AMP-DNM reduces plasma cholesterol levels through promoting fecal cholesterol excretion and not by inhibiting cholesterol absorption [263]. Likely trans-intestinal cholesterol efflux (TICE) contributes to the increased fecal cholesterol secretion. TICE is still a poorly understood process in which cholesterol is exported across the intestine via an unknown mechanism not involving high density lipoprotein [264].

Finally, the impact of AMP-DNM on substrate oxidation and food intake in obese mice was studied [265]. For this, ob/ob mice were orally treated with or without 100 mg/kg AMP-DNM and then next examined in metabolic cages. AMP-DNM treatment markedly improved the obese phenotype. Drug treatment rapidly increased fat oxidation by 129% and decreased carbohydrate oxidation by 35%. Drug administration reduced hepatic triglyceride by 66%. Food intake was decreased by approximately 26%. Exposure to AMP-DNM was found to increase the plasma levels of the appetite-regulating peptide.
YY (PYY) in the ob/ob mice [265]. High doses of N-butyldeoxynojirimycin (NB-DNJ) were also reported to lead to weight loss in healthy lean and leptin-deficient obese (ob/ob) mice through reduced food intake [266]. Lean mice treated with NB-DNJ lost weight in the form of adipose tissue. The mice grew normally without reduction in lean mass. Likewise, obese mice treated with NB-DNJ gained weight at a greatly reduced rate compared to non-treated animals. Food intake was 30% less than in untreated controls in lean and obese mice treated with NB-DNJ. Extracts of mulberry roots and leaves are reported to contain deoxynojirimycin, N-methyl-deoxynojirimycin and 1,4-dideoxy-1,4-imino-D-arabinotol, 1,4-dideoxy-1,4-imino-D-ribitol as well as calystegins (nortropane iminosugars). Of note, mulberry preparations are considered to be useful in the treatment of diabetes type 2 and have also been shown to induce significant weight loss [267-270].

The positive effects on satiety and glucose metabolism suggest a direct role of the brain in the iminosugar regulation of energy metabolism. Nordstrom further extended the investigations by studying the impact of inducible knockout of GCS in hypothalamic neurons (Ugcgf/f//CamKCreERT2) [271]. It was found that neuronal expression of GCS impacts on hypothalamic integration of metabolic signals. A special role is envisioned for gangliosides interacting with leptin receptors (ObR) and modulating leptin-induced signaling metabolites in hypothalamic neurons. Ganglioside-depleted hypothalamic neurons were found to be unable to adapt their activity (measured by c-Fos expression) in response to peripheral energy signals. Mice with inducible forebrain neuron-specific deletion of the GCS gene (Ugcg) display abnormalities such as obesity, hyperthermia, and lower sympathetic activity. Recombinant adeno-associated virus mediated Ugcg expression in the arcuate nucleus (Arc) significantly improved obesity [271]. Next, Herzel et al. reported that the Ugcgf/f//CamKCreERT2 mice show an impaired fasting-induced lipolysis in white adipose tissue (WAT). Hypothalamic insulin receptor (IR) levels and signaling were increased in these mice [272]. In line, the inhibition of GCS in cultured insulin-stimulated hypothalamic cells by GENZ-123346 was found to also increase IR levels as well as IR phosphorylation. This work suggests that glycosphingolipids also modulate hypothalamic IR signaling and protein levels. Future research should clarify if modulation of GlcCer content in the hypothalamus exerts a role in the pleiotropic metabolic effects induced by AMP-DNM. As discussed, a clear picture seems to emerge indicating that excessive ceramide and glycosphingolipids are associated with obesity and contribute to various manifestations of the metabolic syndrome. However, there are also data that at first glance appear conflicting. For example Jennemann and colleagues developed a series of mice with tissue specific inducible knockout of GCS. Animals lacking GCS in their hepatocytes showed no pathology [273]. In addition, specific effects of ceramide in insulin sensitivity regulation have been questioned, since ganglioside formation is closely linked to ceramide availability different views developed on the relative importance of both lipid species in the etiology of insulin resistance. However, a consensus is now reached in which excessive ceramide is thought to cause insulin insensitivity in muscle, whereas excessive ganglioside is the major culprit in adipocytes [274]. Thus, it seems that a GSL-lowering mode of action of AMP-DNM cannot explain satisfactorily all the beneficial metabolic effects observed after AMP-DNM, thus in the last two chapters of the present manuscript we explored alternative mechanisms through which AMP-DNM regulates energy metabolism.

Central regulation of food intake and body metabolism. The brain participates in the direct control of different metabolic responses that modulate feeding and energy expenditure in order to maintain energy homeostasis. The hypothalamus and the brainstem
contain specialized neuronal networks that sense and integrate hormonal, metabolite and neural information, and modulate food intake and energy expenditure in response to altered metabolic conditions [13]. Endocrine signals secreted from different peripheral organs are sensed by anorexigenic (decrease energy intake and increase energy expenditure) and orexigenic (increase energy intake and decrease energy expenditure) neuronal populations that regulate the autonomic nervous system (ANS) through hypothalamic-brainstem circuits. The ANS communicates with peripheral organs and regulates different physiological functions, like adiposity, hepatic glucose production and thermogenesis [275, 276]. The disruption of secretion of endocrine signals or sensing of hormones or metabolites in the hypothalamus and brainstem has been related to the development of obesity and diabetes [13, 276]. In the hypothalamus, the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the dorsomedial nucleus (DMH), the ventromedial nucleus (VMH) and the lateral hypothalamus (LH) present a variety of hormonal and metabolite receptors and transporters that allow a strict monitoring of the peripheral metabolic state [276]. These nuclei maintain neural projections to the lower autonomic centers in the brainstem and spinal cord to modulate the autonomic outflow to peripheral organs [276-278]. In the brainstem, the nucleus tractus solitaries (NTS) not only presents different hormonal receptors, but also sensory vagal terminals that constitute a major neuronal connection between the gut and the brain [13, 279], for example, intestinal lipid sensing can inhibit hepatic glucose production through a vagal-NTS circuit [280]. In particular, nuclei adjacent to circumventricular organs (CVO) (areas of the brain that present an extended vascularization with fenestrated capillaries, allowing a higher diffusion and transport of substances from the general circulation), like the ARC and the NTS, contain neuronal populations that respond to plasma hormone and metabolite concentrations with wider sensitivity thresholds, which make them a blood-brain interface capable of regulating autonomic function [281]. The ARC, adjacent to the CVO median eminence, contains at least 9 different neuronal populations that regulate energy metabolism, in particular 2 populations play an important role for feeding and energy expenditure: the orexigenic Neuropeptide Y (NPY)/Agouti-related peptide (AgRP) neurons and the anorexigenic Proopiomelanocortin (POMC)/Cocaine and amphetamine-regulated transcript (CART) neurons [13, 282]. NPY receptors (Y1 and Y5) in the PVN, DMH and LH of the hypothalamus, but also in limbic areas like the amygdala, septum, hippocampus and accumbens are involved in the metabolic effects observed after intracerebroventricular (icv) NPY administration, which involve increased food intake, decreased stress responses and decreased thermogenesis through inhibition of sympathetic output to the brown adipose tissue (BAT) [283-285]. Similarly, icv administration of AgRP, acting as an inverse agonist of the melanocortin-3 (MC3R) and melanocortin-4 (MC4R) receptors in the PVN and DMH, promotes food intake [286]. Consequently, NPY or AgRP overexpression in the ARC generates an obese and diabetic phenotype in rodents [282, 287]. These observations gave rise to the proposition that the orexigenic action of the ARC was promoted by NPY receptor agonism and AgRP-induced MC3/4R inverse agonism [284, 286]. POMC generates different peptides from the family of melanocortins and opioids after posttranscriptional processing, the majority of which are involved in energy metabolism [286]. α-MSH, the principal anorexigenic peptide produced in ARC POMC neurons, has 2 receptors, MC3R and MC4R, which are present in the PVN, DMH, ARC and limbic areas [288, 289]. α-MSH icv administration diminishes food intake and increases thermogenesis by stimulating sympathetic outflow to the BAT [282, 283]. Accordingly, MC4R KO mice develop hyperphagia, increased adiposity, hyperinsulinemia and hyperleptinemia [286], similar observations have been made
in humans with MC4R mutations, which also develop early-onset obesity [290]. NPY and POMC neurons are highly sensitive to hormonal and metabolite fluctuations in the circulation and act as first-order neurons on which hormones like leptin, insulin, ghrelin and nutrients primarily act [13, 291]. During obesity, exclusively these ARC neurons present leptin and insulin resistance, similar to the insulin and leptin resistance observed in the periphery. Both central leptin and insulin resistance contribute to a worsening of the obese and diabetic phenotypes by impairing satiety, nutrient intestinal absorption, intermediate metabolism and insulin sensitivity, leading to a dysregulation of the energy balance [291, 292]. NPY and POMC neurons receive their metabolic information via the general circulation from hormones produced in the gastrointestinal tract and by afferent fibers coming from the vagal complex in the hindbrain. In the gut, nutrient digestion and absorption of nutrients stimulate the secretion of satiety hormones like cholecystokinin (CCK), peptide Y (PYY) and glucagon like peptide-1 (GLP1) [293]. These hormones are then secreted into the circulation and detected by the hypothalamus to modulate satiety and decrease postprandial hepatic glucose production [276]. For example, GLP1 is produced by intestinal L-cells after a meal. After secretion, GLP1 acts primarily on the GLP1 receptor (GLP1r) on vagus nerve terminals [294, 295], but also on GLP1r in the ARC, PVN and DMH of the hypothalamus, in limbic areas and in the caudal brainstem [295]. Brain GLP1r is involved in the stimulation of satiety and inhibition of gastric emptying induced by GLP1 [295, 296]. Another area highly sensitive to the peripheral metabolic state is the NTS of the brainstem, close to the CVO area postrema. The NTS is perfectly located for sensing humoral signals from the circulation and receiving neural signals from the gastrointestinal tract and liver through the sensory vagus nerve [293, 297]. It contains NPY and POMC neuronal populations, but also neuronal populations that secrete hormones and peptides similar to the ones produced in the gastrointestinal tract [13, 276]. As the hypothalamus, the NTS presents numerous hormonal receptors that allow its neuronal populations to sense and respond to changes in hormones and metabolites in the circulation. One neuronal population that has attracted a lot of attention, is the preproglucagon (PPG) neuronal population in the NTS, the precursor peptide of the appetite-regulating GLP1. Exenatide and Liraglutide, agonists of the GLP1r, have been proven beneficial for the treatment of type 2 diabetes and body weight loss [298], their beneficial effects have been partially explained by a direct action on the GLP1r located in the brain [299]. NPY, POMC and GLP1 neurons of the NTS maintain reciprocal projections with the PVN, LH, DMH and ARC in the hypothalamus in order to regulate energy metabolism [13, 279, 297, 300]. The NTS is also a sensory gate of chemosensory information from the peripheral organs to the rest of the brain. For example, taste-related information is transmitted from the taste buds in the tongue to the NTS through the cranial nerves [301]. Sweet, bitter and umami G-protein-coupled taste receptors (GPC-TRs) and sour and salty ion channels of the transient receptor potential expressed on the apical end of taste receptor cells in the oral cavity are activated by different tastants in the food. This information is further transmitted to the NTS, the first gustatory relay center in the brain, further projections run to the hypothalamus, amygdala, thalamus and to the gustatory and prefrontal cortex to integrate and combine chemosensory and nutritional information to regulate feeding behavior [302, 303]. In view of the role of the brain in energy metabolism regulation, in the last two chapters of the present manuscript, we explored the possibility that AMP-DNM acts on the CNS to exert its metabolic beneficial effects. In view of its bitter taste a role for interaction between iminosugars and the gustatory system is also evaluated.
Figure 4. Schematic representation of the hypothalamic and brainstem structures involved in the regulation of peripheral energy metabolism. Based on Yi, et al [276].
Outline and goals of the thesis.

This thesis concerns different investigations of brain structures and brain mechanisms connected to or affected by glycosphingolipids. In part 1 the location of GlcCer and Gal-Cer degrading lysosomal and non-lysosomal glycosidases is visualized in an unprecedented manner with fluorescent mechanism-based cyclophellitol-type probes. In addition, experimental evidence is presented for a detrimental role of GBA2 in the loss of motor neurons in mice suffering from Niemann-Pick disease type C, a lysosomal storage disorder. The section is closed with a comparative review of Gaucher disease and Fabry disease, two biochemically related but clinically diverse lysosomal storage disorders. Part 2 describes investigations with normal and obese rodents. Investigations focused on changed expression of sweet and bitter taste receptors during obesity and the complex mechanism by which oral administration of the hydrophobic iminosugar AMP-DNM, a known modulator of GlcCer metabolism, positively impacts the central regulation of satiety and body metabolism in obese animals.

Part 1 comprises six chapters.

Chapter 1 builds on the earlier development of activity-based probes labeling either selectively GBA (cyclophellitol-epoxide type ABPs) or GBA and GBA2 together (cyclophellitol-aziridine type ABPs). Intravenous administration of the BODIPY-tagged ABPs does not yield labeling of enzymes in the brain due to poor brain penetrance of the probes. The interest in the location of GBA and GBA2 in the brain is great given the neuropathology in GBA-deficient patients with severe GD and the presumed role of GBA in Parkinsonism, as well as the neurological manifestations of patients deficient in GBA2. Described is the development of a suitable method to visualize active GBA and GBA2 molecules in the brain of rats, based on sequential intracerebroventricular administration of the two kinds of ABPs. Active GBA is most prominently detected in brain areas related to motor control (basal ganglia and brainstem structures), while active GBA2 is mostly detected in the cerebellar cortex with ABP labeling.

Chapter 2 describes an investigation on β-galactosyl configured cyclophellitol-epoxide tagged with a fluorophore. It is shown that this probe selectively labels galactocerebrosidase (GALC), the enzyme deficient in Krabbe disease patients and Twitcher mice. The specificity of the probe makes it an interesting tool to employ in fundamental research on GALC and diagnosis of Krabbe disease patients. Demonstrated is the imaging of active GALC in mouse brain following intracerebroventricular administration of the ABP to the animals.

Chapter 3 reports on the specificity of β-glucopyranosyl-configured cyclophellitol-aziridine ABPs. It is demonstrated that at very high concentrations these ABPs covalently label multiple β-glucosidases next to the two β-glucosidases GBA and GBA2. This phenomenon can be exploited for simultaneous labeling of multiple glycosidases employing a combination of available ABPs with different fluorophores and specificity. It is shown that various β-glucosidases and β-galactosidases (GBA, GBA2, GALC, GLB1 (lysosomal acid β-galactosidase)) can in parallel be differentially visualized with SDS-PAGE. Application of this procedure for diagnostic purposes is discussed.

Chapter 4 describes the use of ABPs to follow in a head-to-head comparison the uptake by cultured cells of two therapeutic recombinant GBA preparations, velaglucerase and imiglucerase differing in N-glycan composition. Moreover, the bodily distribution
of both ABP-labeled enzymes upon simultaneous intravenous administration in mice is reported. No significant differences were found between the two enzymes, in contrast to earlier claims.

Chapter 5 concerns an investigation on the role of GBA2 in the loss of motor coordination in Niemann Pick type C (NPC) mice. It is demonstrated that genetic absence of GBA2 and its pharmacological inhibition both increase life span by slowing down Purkinje cell loss. The bifacial effect was reached while lysosomal glycosphingolipid storage was not reduced, illustrating nicely the role of cellular adaptations in metabolism during pathogenesis.

Chapter 6 presents a review of clinical and biochemical similarities and differences of the glycosphingolipidoses Gaucher disease and Fabry disease. Particular attention is devoted to metabolite and protein biomarkers for the two lysosomal storage disorders. Reliable laboratory diagnosis of both diseases is of great importance, particularly since the availability of costly therapies for these conditions.

Part 2 exists of two chapters.

Chapter 7 deals with an investigation of the expression of bitter and sweet receptors in normal and obese mice. The study was prompted by clues that the hydrophobic iminosugar AMP-DNM, the topic of research in the concluding chapter, might have a very bitter taste. Sweet and bitter receptors are documented to promote craving for and avoidance of food, respectively. Since satiety is abnormal in obese mice the impact of obesity on expression of sweet and bitter GPCTRs, as well as the α-subunits of the associated signalling complex, in gustatory and non-gustatory tissues was studied with emphasis on hypothalamus and brainstem areas involved in energy homeostasis. It is shown that many of the GPCTRs and α-subunits are co-expressed in these brain areas and that obesity decreases expression of T1R3, T2R116, Gα14, αTrans and TRPM5. Obesity was found to prominently reduce the expression of most bitter and sweet receptors in the duodenum. In conclusion, obesity influences components of sweet and bitter taste sensing in the duodenum as well as regions of the mouse brain involved in energy homeostasis.

Chapter 8 describes a study on the mode of action of orally administered AMP-DNM in obese mice. It has been already shown that AMP-DNM improves insulin sensitivity and glycemic control, protects against loss of pancreatic beta-cells, reduces inflammation in adipose tissue and liver, stimulates beta-oxidation of fats and counteracts hepatosteatosis, reduces hyperlipidemia and hypercholesterolemia and prevents atherosclerosis. Moreover, it was found that AMP-DNM treatment corrects satiety, even in obese animals lacking leptin or the leptin receptor. The latter finding stimulated the detailed investigation of the effect of AMP-DNM on the central regulation of satiety and body metabolism. Oral AMP-DNM was found to activate POMC neurons in the hypothalamus and GLP1 neurons in the brainstem, both known to reduce appetite. Oral AMP-DNM administration was also found to increase plasma GLP1. GLP1 receptor (GLP1r) deficient mice showed no AMP-DNM corrections in satiety but the respiratory exchange ratio was nevertheless reduced pointing to increased utilization of fat as energy source. Mice with a conditional knock out of GLP1r in the hypothalamus showed no reduced food intake upon AMP-DNM treatment, but still improved glucose tolerance. AMP-DNM was found to activate brown adipose tissue of the exposed animals. Analysis of animals lacking GLP1r totally or specifically in the hypothalamus indicated that GLP1 signaling in the hypothalamus is essential for the satiety improving effect of AMP-DNM, as well as its
activation of brown adipose tissue. Cultured enteroendocrine cells with expressed bitter receptor hT2R16 and T2R118 respond to AMP-DNM by secretion of GLP1. In conclusion, oral administration of AMP-DNM to obese mice promotes secretion of GLP1 by enteroendocrine cells following recognition by the bitter receptor T2R118, the mouse analogue of hT2R16. GLP1 subsequently promotes the central regulation of food intake and brown tissue activation, but it is not essential for improved glucose homeostasis or increased beta-oxidation of fats in the body of obese mice.

The **Summary** offers an overview of the various studies presented in this thesis.

The **Discussion** deals with the implications of the findings of the presented investigations. These are discussed while taking into account recent literature and insights, additionally it proposes new mechanisms for AMP-DNM action on metabolism. Finally, future directions of research are outlined.

**References**


