MINI-REVIEW

Simplifying complexity: genetically resculpting glycosphingolipid synthesis pathways in mice to reveal function

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Abstract Glycosphingolipids (GSLs) are a group of plasma-membrane lipids notable for their extremely diverse glycan head groups. The metabolic pathways for GSLs, including the identity of the biosynthetic enzymes needed for synthesis of their glycans, are now well understood. Many of their cellular functions, which include plasma-membrane organization, regulation of cell signaling, endocytosis, and serving as binding sites for pathogens and endogenous receptors, have also been established. However, an understanding of their functions in vivo had been lagging. Studies employing genetic manipulations of the GSL synthesis pathways in mice have been used to systematically reduce the large numbers and complexity of GSL glycan structures, allowing the in vivo functions of GSLs to be revealed from analysis of the resulting phenotypes. Findings from these studies have produced a clearer picture of the role of GSLs in mammalian physiology, which is the topic of this review.

Keywords Glycosphingolipids · Glycosyltransferases · Gangliosides · Mouse models · Gene targeting

Introduction

Glycosphingolipids (GSLs) constitute the most structurally diverse subgroup of the sphingolipid family [1]. They contain a hydrophilic glycan head group, consisting of one or more oligosaccharide residues, and a hydrophobic ceramide anchor. The GSLs reside primarily in plasma membranes, with the ceramide anchor embedded in the lipid bilayer and the oligosaccharide head group exposed to the extracellular environment.

Dedicated GSL synthesis is initiated by the transfer of either a glucose or a galactose moiety in β-linkage to the position 1 hydroxyl group of ceramide, resulting in the formation of glucosylceramide (GlcCer) or galactosylceramide (GalCer) (Fig. 1). The subsequent addition of galactose to GlcCer forms lactosylceramide (LacCer). LacCer serves as a hub in the pathway in which the substrate can be diverted into one of several pathways by the actions of distinct glycosyltransferases that lead to the formation of GSL subfamilies, with distinct core or root carbohydrate sequences, known as GSL series (Fig. 1). GalCer is also modified similarly subsequent to its synthesis, but the complexity the GalCer-based GSLs (gala series) is far less than that of the GlcCer-based GSLs.

When the oligosaccharide head groups of GSLs are modified by the addition of one or more sialic acids (usually α-2,3- or α-2,8-linked N-acetylneuraminic acid), the GSLs produced are termed gangliosides [2] (Fig. 1). GSL head groups can also be modified by sulfate and are called sulfatides. All told, the number of GSLs with distinct oligosaccharide structures has been estimated to be greater than 400 [1].

Some of the mechanisms by which GSLs exert their functions at a cellular and molecular level have been well defined [1, 3–5]. Their most basic function is as building blocks of the plasma membrane. In this case, GSLs, due to their ability to form liquid-ordered phases in membranes, can drive the formation of functionally and structurally discrete plasma-membrane domains [3, 6]. GSLs, through their glycan head groups, can act as binding sites for pathogens and toxins [7] and, in a similar manner, for endogenous molecules, such as myelin-associated glycoprotein, to facilitate cell-to-cell interactions [8]. Through lateral interactions with membrane proteins, such as the insulin and epidermal growth factor receptors, GSLs can modulate the strength of receptor-mediated signaling pathways [9, 10]. Finally, they can function
GSLs' functional diversity. This hurdle has been overcome by the systematic elimination of diverse GSL glycan structural groups in genetic deletion studies. Through the targeting of specific glycosyltransferases and other modifying enzymes, either singly or in combination and in a tissue-specific fashion—in effect simplifying the complexity of the GSLs—their in vivo functions have been elucidated from analysis of the resulting phenotypes.

Global deletion of glucosylceramide-derived GSLs (Ugcg knockout)

UDP-glucose:ceramide glycosyltransferase (GlcCer synthase) is the rate-limiting enzyme for GlcCer-based GSL biosynthesis. It is encoded by the Ugcg gene in the mouse [12, 13]. GlcCer synthase catalyzes the synthesis of GlcCer by the transfer of a glucose residue from UDP-glucose to ceramide. Once formed, GlcCer is modified by glycosyltransferases in the Golgi, ultimately giving rise to hundreds of more complex GSLs [1] (see Fig. 1).

Global deletion of the Ugcg gene in mice, which consequently eliminates all GlcCer-based GSLs, caused early embryonic lethality [14]. Ugcg knockout (KO) embryos developed until gastrulation at E6.5, forming the ectodermal, endodermal, and mesodermal layers, and with embryonic patterning. A day later, mutant embryos showed retarded growth and extensive apoptosis, primarily in the ectodermal layer, and were subsequently resorbed. These results are a vivid demonstration that GlcCer-based GSL synthesis is an absolute requirement for mammalian embryonic development [15].

Pan nervous-system deletion of GlcCer GSLs

GSLs, primarily in the form of gangliosides, are abundantly expressed in the nervous system, making up about 10% of the total lipids and suggesting a vital role during the formation of and/or function in these tissues [5, 16]. In order to determine the importance of these lipids during the formation of a functional nervous system, two independently derived lines of mice lacking Ugcg in neuronal and glia cells were established by combining a floxed Ugcg allele and a Cre recombinase transgene under the control of the pan nervous system rat nestin promoter and enhancer (NesCre) [17, 18]. NesCre-Ugcg KO mice did not show evidence of embryonic lethality and appeared normal at birth. However, they developed neurologic
abnormalities over time. The severity of the phenotypes in the two lines was different and appeared to be related to the level of residual Ugcg expression found in the respective NesCre-Ugcg KO brains. The mouse line produced by Jennemann et al. [18], in which the levels of Ugcg mRNA and gangliosides in brain were nearly completely eliminated, died by 3 weeks of age, and displayed very severe neurological symptoms, including ataxia, reduced axonal branching of neurons, and disturbed myelination of peripheral nerves. The line generated in the Proia laboratory [17] showed residual Ugcg expression, a significant, but incomplete reduction of ganglioside levels in the brain, as well as a longer life span. These mice also displayed abnormal gait, along with a progressive loss of Purkinje cells [18].

These reports show that GlcCer-based GSL synthesis is expendable for embryonic development of the nervous system, but is required for its proper development, stability, and function after birth.

Neuronal-specific deletion of GlcCer-based GSLs

The deletion of Ugcg specifically in neuronal cells was achieved by generating floxed Ugcg mice expressing the Cre recombinase gene under the L7 promoter only in Purkinje cells [19]. These mice, showed Purkinje cell degeneration at about 3–4 months of age, which was characterized by axon swelling and accumulation of axonal transport cargos prior to Purkinje cell loss. Significant abnormal myelination was present, as were detached paranodal junctions (the attachment sites of neuronal axons and oligodendrocytes), implying that the neurally synthesized GSLs were critical for these axonoglial interactions.

In a separate study, Ugcg was deleted after birth using an inducible Cre recombinase (Cre-ERT2) under the control of the neuronal calcium/calmodulin-dependent kinase II α promoter (CamK). In these mice, expression of the Ugcg gene was deleted from the neurons of the forebrain, including the hypothalamic nuclei, which are involved in energy homeostasis regulation. CamKCre-ERT2-Ugcg KO mice showed progressive body-weight gain, hypometabolism, and hypothermia. This phenotype could be reversed by virus-mediated expression of Ugcg in the arcuate nucleus (Arc) of the hypothalamus, a key regulator of feeding and metabolism. Leptin signaling, which is essential for metabolic homeostasis, was impaired in Arc neurons from the CamKCre-ERT2-Ugcg KO mice. The basis for the impaired leptin responsiveness in the Arc neurons appeared to be due to a lack of GlcCer-based gangliosides, which normally directly associate with the leptin receptor on the plasma membrane and promote leptin responsiveness [20].

Oligodendrocyte-specific deletion of GlcCer-based GSLs

Ugcg was deleted specifically in oligodendrocytes by crossing floxed Ugcg mice with a line expressing Cre recombinase under the control of the myelin-associated enzyme 2′, 3′ cyclic nucleotide 3′ phosphodiesterase (CnpCre) [21]. The resulting oligodendrocyte-specific CnpCre-Ugcg KO mice did not show any myelin abnormalities, indicating that oligodendrocyte GlcCer-derived GSLs, unlike those produced by neuronal cells, are not essential for myelin structure and stabilization.

Keratinocyte-specific deletion of Glc-Cer based GSLs

Keratinocytes produce large amounts of GSLs, mainly GlcCer, accounting for almost 4% of the total lipid content in the epidermis, which is packed into lamellar bodies and delivered to the stratum corneum for further processing to generate ceramide [22, 23]. Floxed Ugcg mice were crossed with a keratin K14 promoter-driven Cre line to produce mice lacking Ugcg expression in keratinocytes (K14Cre-Ugcg KO mice) [24]. K14Cre-Ugcg KO pups showed profound desquamation and epidermal dehydration and died at about 4 days of age. Their skin showed broadened epidermal layers, abnormal lamellar bodies, keratinocyte apoptosis, and inflammation. When epidermal deletion was induced after birth using a tamoxifen-inducible (TAM) K14Cre transgene to bypass the early lethality, TAM-K14Cre-Ugcg KO mice showed epidermal abnormalities similar to the K14Cre-Ugcg KO mice [25]. These studies supported the conclusion that Ugcg expression is essential for the formation of a functional epidermal permeability barrier through the generation of GlcCer, a precursor for the formation of ceramide in the skin.

Liver-specific deletion of Glc-Cer based GSLs

Liver is a central site controlling sphingolipid metabolism [26]. A liver-specific knockout of Ugcg was generated by crossing floxed Ugcg mice with mice expressing Cre recombinase under the control of the albumin promoter (AlbCre-Ugcg mice) [27]. The mice had greatly reduced GSL levels in liver and plasma, pinpointing liver as the major source of plasma GSLs. No other phenotype was detected in the AlbCre-Ugcg mice, even after challenge with a high-fat diet, arguing against a vital role of hepatic GlcCer synthesis in glucose control, liver steatosis, or cholesterol metabolism.

Enterocyte-specific deletion of Glc-Cer based GSLs

GSLs are highly concentrated in the columnar epithelial cells that form the villi in the intestine, known as enterocytes.
Enterocyte-specific Ugcg-deficient mice were generated by crossing floxed Ugcg mice with mice expressing Cre recombinase under the control of the villin promoter induced either in the embryo (using VilCre-expressing mice) or during adult life (using VilCre-ERT2-expressing mice) [28]. GlcCer-derived GSLs were profoundly absent in the intestines of the VilCre-Ugcg pups. Although undistinguishable from control littermates at birth, VilCre-Ugcg pups did not gain weight and died by postnatal day 8. VilCre-Ugcg pups showed normal polarization of the enterocytes, but had abnormal mucosal villi, decreased fat deposits, and a severe decrease in the ability of enterocytes to uptake lipid. Inducible VilCre-ERT2-Ugcg mice showed an almost complete absence of intestinal GSLs 4 days after deletion induction, which was concomitant with significant structural alterations in the villous epithelia. These mice also had decreased body weight and reduced lipid absorption by the enterocytes. These findings demonstrate that GlcCer-derived GSLs are necessary for proper intestinal function.

Deletion of lactosylceramide-based GSLs: B4gal5 KO

LacCer is synthesized in the Golgi lumen by LacCer synthase (β-1,4-galactosyltransferase 5) via the transfer of galactose from UDP-galactose to GlcCer. B4gal5 null mice were generated by conventional gene targeting [29] and through a large-scale gene-trap project [30]. Both gene targeting strategies led to lethality in utero. The B4gal5−/− embryos appeared normal until E6.5, showed retarded growth by E7.5, and died around E10.5. B4gal5−/− embryos showed bleeding in the extra-embryonic tissues at E7.5 [29]. The early lethality of the B4gal5−/− embryos could be rescued by wild-type extra-embryonic tissues; however, these embryos did not develop past E18.5, indicating that B4gal5 has a direct role in later embryonic development within the embryo proper [29].

Deletion of ganglio-series GSLs: B4galnt1 KO

GM3 (CMP-sialic acid:lactosylceramide α-2,3-sialyltransferase) is encoded by St3gal5. The enzyme transfers sialic acid to LacCer to produce GM3, the simplest of the sialic acid-containing GlcCer-based gangliosides and a precursor for complex gangliosides [2]. St3gal5-deficient mice had normal life span [39]. Analysis of their ganglioside content demonstrated the absence of GM3 and all GM3-derived gangliosides from the a- and b-series (Fig. 1), which are the major gangliosides in the brain [2].

GM3 synthase deletion increased insulin sensitivity; that is, St3gal5−/− mice had enhanced phosphorylation of the insulin receptor in skeletal muscle after insulin binding, and better responses in glucose- and insulin-tolerance tests. Accordingly, they were also protected from insulin resistance development after a high-fat diet regimen [39]. Diabetic diet-induced obese St3gal5−/− mice were also found to be protected from a wound-healing defect. Wound healing generally involves proliferation and migration of keratinocytes, which are both mediated by the insulin receptor and insulin growth factor-1 receptor. Because diabetic obese St3gal5−/− mice indeed had activated insulin receptor and insulin growth factor-1 receptor signaling, particularly in the presence of high glucose, the wound-healing defects observed in wild-type diabetic obese mice were obviated [40].
An independently generated line of St3gal5−/− mice exhibited complete hearing loss [41]. The St3gal5−/− mice showed degeneration and disappearance of the organ of Corti, which is the hearing sensor in the cochlea. These results suggest that GM3 synthesis is essential for the early maturation of the cochlea and that it is important in development of the auditory system.

Deletion of b-series gangliosides: St8sia1 KO

GD3, the precursor of the di-sialo b-series gangliosides (Fig. 1), is generated by the addition of sialic acid to GM3 by GD3 synthase (CMP-sialic acid: GM3 α-2,8-sialyltransferase), encoded by the St8sia1 gene. Despite the absence of the normally prominent b-series gangliosides in the brain, the deletion of the St8sia1 gene did not cause apparent developmental abnormalities in the nervous system [42, 43]. Adult St8sia1−/− mice were found to be viable and grossly normal.

A second independently derived line of St8sia1−/− mice exhibited reduced regeneration capacity of the hypoglossal nerve, showing fewer surviving neurons after resection of the nerve [42]. In addition, St8sia1−/− mice displayed increased sensory responses to thermal and mechanical stimuli and decreased response to subcutaneous formalin injection, which is a measure of clinical pain, suggesting GD3-based gangliosides mediate transmission of pain and might be important in the sensory system [44].

“GM3-only” mice: St8sia1/B4galnt1 double knockout

St8sia1/B4galnt1 double knockout (DKO) mice were generated by crossing animals carrying the St8sia1 and B4galnt1 null alleles [43]. Homozygous DKO mice were viable and expressed only GM3 as a GlcCer-based ganglioside in their brains. Strikingly, these DKO mice were extremely susceptible to sound-induced lethal seizures, and most died by 3 months of age [43]. A separate DKO line did not display any seizure-related phenotype, although these mice had decreased weight, motor dysfunction, low fear stress, and reduced sensory responses due to peripheral nerve degeneration [45]. These DKO mice also acquired severe skin lesions, possibly caused by the reduced sensory function [46]. These findings show that complex ganglioside expression is essential to maintain the normal function of the central and peripheral nervous systems, and that these functions of complex ganglioside structures cannot be replaced by the expression of the simple ganglioside GM3.

“Ganglioside-less” mice: St3gal5/B4galnt1 DKO

Phenotypes seen in B4galnt1−/− or St8sia1/B4galnt1 DKO mice demonstrated that expression of even simple gangliosides such as GM3 and GD3 were sufficient for the development of a functioning nervous system, although with some defects. To answer if gangliosides were truly essential for nervous-system development, the elimination of all GlcCer-based gangliosides was achieved by crossing mice carrying null mutations in the St3gal5 and B4galnt1 genes (Fig. 1) to create St3gal5/B4galnt1 DKO mice. These DKO mice were initially viable but soon developed hindlimb weakness, tremors, and ataxia; most died within 3 months of birth [47]. As expected, and in accordance with the proposed biosynthetic pathway, these DKO mice were unable to synthesize any gangliosides of the ganglio-series and instead accumulated LacCer. DKO mice showed severe neurodegenerative symptoms, including a smaller brain and prominent areas of vacuolization in the spinal cord and brain white matter. Myelinated axons were degenerated in these mice, and showed abnormalities of paranodal junctions at the nodes of Ranvier, which suggests impairment of the axon-glia interactions. These findings indicate that GlcCer-based gangliosides are not required for early neural differentiation or morphogenesis of the brain, but are critical for promoting the formation or stabilization of functional axon-glia interactions. The absence of gangliosides ultimately leads to axonal degeneration.

Deletion of the globo-series of GSLs: A4galt KO

Gb3 synthase (α-1,4-galactosyltransferase), which is encoded by the A4galt gene, is responsible for the synthesis of Gb3 (Galα1–4Galβ1–4Glcβ1Cer) by the addition of an α-1,4-linked galactose to LacCer, initiating the production of the globo-series of GSLs. A4galt−/− mice were deficient in Gb3 and other globo-series GSLs [48]. These KO mice appeared normal and lacked any obvious phenotype. Gb3 has been reported to be a ligand for the bacterial endotoxins Shiga-like and verotoxins, which are associated with hemorrhagic colitis and hemolytic uremic syndrome. Accordingly, A4galt−/− mice were found to be protected from the effect of these toxins, clearly demonstrating that globo-series GSLs are endogenous ligands for these toxins [48].

Deletion of the isoglobo-series of GSLs: A3galt2 KO

iGb3 synthase (α-1,3-galactosyltransferase), which is encoded by the A3galt2 gene, initiates the formation of the isoglobo-series of GSLs with the synthesis of iGb3 (Galα1–3Galβ1–4Glcβ1Cer) from LacCer by the addition of an α-1,3-linked galactose (Fig. 1). Deletion of the A3galt2 gene
Deletion of the lacto/neolacto-series of GSLs: B3gnt5 KO

Lc3 synthase (β-1,3-N-acetylglucosaminyltransferases 5), which is encoded by B3gnt5, produces Lc3 (GlcNAcβ1–3Galβ1–4Glcβ1Cer), initiating the lacto/neolacto-series of GSLs (Fig. 1), with the transfer of N-acetylglucosamine to LacCer. Three independent groups have reported targeting the B3gnt5 gene in mice, with different outcomes. Genetic deletion of the B3gnt5 gene in mice by Biellmann et al. [51] caused embryonic lethality at the preimplantation stage, possibly due to defects in cell-cell adhesion that contribute to embryo compaction and implantation. The line generated by Togayachi et al. produced viable B3gnt5−/− mice that showed a defect in B-cell signaling due to abnormal raft formation [52]. Kuan et al. [53] developed B3gnt5−/− mice by two different strategies. They reported that B3gnt5−/− mice showed splenomegaly and enlarged lymph nodes, and had shorter, variable life spans. They also had lower B-cell numbers, exhibited hair loss, were obese, and had reproductive defects. The reasons for the discrepancies among the phenotypes of the B3gnt5−/− mouse lines in the three reports are not apparent.

Deletion of sulfated GalCer: Gal3st1 KO

Gal3st1 encodes for the glycolipid-specific GalCer sulfotransferase [58], which adds a sulfate group to GalCer to form SM4 which is incorporated into myelin (Fig. 1) [59–61]. To determine the role of sulfated GSL in vivo, mice with a deletion of Gal3st1 were generated [62]. Gal3st1−/− mice were normal at birth, but as they aged they developed neurological symptoms (exhibiting as weakness of the hindlimbs, tremors, and progressive ataxia) However, these mice were able to survive for more than 1 year. These mice lacked SM4, but still expressed GalCer in the brain [62]. Their axons were myelinated, but the myelin showed vacuolation, and exhibited defective paranodal junctions and abnormal ion-channel localization along the axon membrane [62–65]. These results demonstrate the important role of sulfated GalCer in the formation interactions between axons and glia.

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Sulfated GSLs are highly enriched in the kidney, especially at the distal nephron segments and in the renal medulla [61, 66, 67]. To determine the role of sulfated GSLs specifically in kidney, Gal3st1 was deleted via renal epithelia-specific genetic disruption either individually to eliminate sulfated GSLs, or in combination with Uggg to further remove possible compensatory anionic GlcCer-based GSLs, Mice carrying floxed Gal3st1 and Uggg alleles were crossed with mice expressing the Cre recombinase gene under the control of the tubular epithelial cell-specific paired box gene 8 promoter (Pax8Cre) [68]. Kidneys from single (KO) and double KO (DKO) mice lacked the predicted sulfated GSL species in the kidney and showed generally normal kidney morphology. However, the Pax8Cre-Gal3st1 KO and Pax8Cre-Gal3st1/ Uggg DKO mice both showed significantly lower pH and less ammonium excretion in the urine. When fed an acidic diet, the Gal3st1 KO mice exhibited decreased ammonium accumulation in the papilla, and chronic hyperchloremic acidosis. The results are consistent with the notion that sulfated GSLs in the kidney act as counterions of interstitial NH₄⁺, allowing its accumulation in the kidney papilla and enabling sufficient renal excretion of protons to maintain a proper blood pH [68]. The results show that sulfated GSLs are critical for physiologic acid-base homeostasis.

Conclusions and perspectives

During the period of time since the description of the first knockout mice targeting enzymes in the GSL synthesis pathway in 1996 [32, 55], we have seen a striking increase in our understanding of the roles of GSLs in physiology (Fig. 2). We have learned that they are not only fundamentally important for embryonic development, but also have key tissue-specific functions, including formation of the skin permeability barrier, acid handling in the kidney for maintenance of acid–base homeostasis, and key nervous-system functions (including in neuron-glial interaction to maintain myelin stability and neuronal control of appetite). It is likely that we have only scratched the surface of GSL functions, given the existence of hundreds of different GSL structures and that we only understand the function of a relatively very few. Furthermore, our knowledge of how GSLs act in human disease is only in its infancy. While lysosomal storage diseases in which GSL degradation pathways are disrupted have been known for many years [69], only recently have genetic diseases involving the GSL synthesis pathways been described [70–73]. A new challenge is to build on current insights into normal GSL functions (Fig. 2) to understand how GSLs play a role in the pathogenesis of other disorders. In this vein, significant progress is already being made in a number of human diseases [74–76]. These insights will eventually lead to therapeutics aimed at targeting specific GSL pathways for the treatment of these disorders.

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Conflict of interest The authors declare that they are free from conflict of interest.

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