Glycosphingolipid functions: insights from engineered mouse models

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Glycosphingolipids are remarkable for their structural complexity and their distinctive patterns that mark different tissues and stages of development. The physiological functions of glycosphingolipids are likely to be profound and are only beginning to emerge. Much of this new information is due to the study of mutant mice lacking specific sets of glycosphingolipids, the topic of this review.

Keywords: gangliosides; glycosphingolipids; knockout mice

1. INTRODUCTION

Glycosphingolipids are present on all mammalian cell plasma membranes. These are amphipathic molecules consisting of a ceramide lipid anchor linked to an oligosaccharide chain of variable length and composition (Kolter *et al.* 2002; Sandhoff & Kolter 2003). The most widely distributed glycosphingolipids contain glucosylceramide as a core structure. These include the gangliosides, which are defined as glycosphingolipids that contain one or more sialic acid residues. Gangliosides with glucosylceramide as a precursor are highly concentrated on the surface of neurons but are also widely distributed among different cell types (Sandhoff & Kolter 2003).

Their great structural complexity and remarkable patterns of distribution marking stages of development and cell types has fostered the concept that glycosphingolipids must play vital roles in many biological processes. Until recently, their physiological functions had been very difficult to address. Most investigations that were performed consisted of addition of the glycosphingolipids, their genes or inhibitors of their synthesis to cells in culture. Such experiments, while valuable at a cellular level, could not adequately address the physiological roles of glycosphingolipids at more complex levels. Recently, with the advent of targeted mutagenesis in the mouse, it has become possible to eliminate specific groups of glycosphingolipids in the complex setting of a whole organism. This review will summarize some of these findings.

2. BIOSYNTHESIS

The biosynthetic pathway of glucosylceramide-based glycosphingolipids and gangliosides begins in the endoplasmic reticulum with the synthesis of ceramide by serine palmitoyl transferase (Kolter *et al.* 2002; Sandhoff & Kolter 2003). After transfer to the Golgi apparatus, ceramide is modified by the action of glucosylceramide syn-

thase (UDP-glucose:ceramide glucosyltransferase) with the transfer of glucose from UDP-glucose to produce glucosylceramide on the cytosolic surface of the Golgi (figure 1). Glucosylceramide is transferred to the luminal leaflet of the Golgi where it is then modified by the addition of a galactose moiety to produce lactosylceramide. Subsequent attachment of a sialic acid yields GM3, the first ganglioside produced in this pathway and a critical branch point in the ganglioside synthesis (figure 1). GM3 ganglioside can be modified by β -1,4-*N*-acetylgalactosaminyltransferase (GM2 synthase/UDP-GalNAc:lactosylceramide/GM3/GD3) to produce the a-series ganglioside GM2. This step is necessary for the synthesis of complex gangliosides. Alternatively, GM3 can be acted on by GD3 synthase (CMP-sialic acid:GM3 α -2,8-sialyltransferase), which switches the synthesis pathway to the b-series (figure 1).

After their synthesis is completed in the Golgi, glycosphingolipids are delivered to the plasma membrane where they are organized into lipid-based microdomains termed rafts and caveolae (Simons & Toomre 2000; Harder 2003). The ability of the ceramide anchor in sphingolipids to associate with cholesterol is believed to drive the assembly of these structures. These lipid microdomains are thought to function as signalling platforms by virtue of their affinity for signalling proteins—transmembrane receptors, GPI-anchored proteins, RhoA, G-proteins and Src kinases (Hakomori 2002; Harder 2003). Glycosphingolipids are important components of these assemblies as evidenced by the finding that exogenous administration of gangliosides displaced GPI-anchored proteins from rafts (Simons *et al.* 1999). Furthermore, TAG-1, a GPI-anchored neuronal cell adhesion molecule, was found to be complexed with GD3 ganglioside and the Src family kinase, Lyn, within lipid microdomains (Kasahara *et al.* 1997, 2000; Prinetti *et al.* 2001).

3. GLUCOSYLCERAMIDE SYNTHASE

The first committed biosynthetic step for the vast majority of glycosphingolipids as well as for most gangliosides is accomplished by glucosylceramide synthase. The gene that encodes glucosylceramide synthase, *Ugcg*, was

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Figure 1. Biosynthetic pathway of gangliosides. The steps blocked in mutant mice are illustrated. *Ugcg*: gene encoding glucosylceramide synthase; *GalNAcT*: gene encoding β -1,4-*N*-acetylgalactosaminyltransferase. *GD3S*: gene encoding GD3 synthase. Key: red squares, glucose; green squares, galactose; orange circles, GalNAc; yellow triangles, 2,3-NeuAc; dark blue triangles, 2,8-NeuAc; light blue triangles, 2,6-NeuAc.

found to be expressed during preimplantation development as early as the eight-cell stage (Yamashita *et al.* 1999). When mice heterozygous for a disrupted *Ugcg* gene were mated, no offspring homozygous for the mutation were obtained, suggesting embryonic lethality (Yamashita *et al.* 1999). Indeed, timed matings revealed normal-appearing embryos only up until embryonic day (E) 6.5. At E7.5, during gastrulation, the homozygous mutant embryos appeared smaller than controls. By E8.5 all mutant embryos were dead.

Histological analysis of the *Ugcg*^{-/-} embryos revealed the presence of the three primitive germ layers—ectoderm, mesoderm and endoderm (Yamashita *et al.* 1999). However, the overall embryonic structure was disturbed with a reduced extraembryonic portion relative to the embryonic portion. *Brachyury* (T gene) expression, an early mesodermal marker, was present, confirming that gastrulation was occurring. The smaller size of the mutant embryos was not due to impaired growth. Instead, the small size and death of the embryos was the result of a massive apoptotic process centred in the ectodermal layer.

To determine if formation of highly differentiated cells and tissues was possible in the absence of glycosphingolipids, embryonic stem cells were established with both alleles of *Ugcg* disrupted (Yamashita *et al.* 1999). These cells were devoid of glucosylceramide activity and were deficient in glycosphingolipids. When injected into subcutaneous sites in mice, the mutant and the wild-type cells formed teratoma tumours with similar kinetics. However, when examined histologically, the mutant teratomas were found to lack well-differentiated cell types—muscle, smooth muscle, cartilage and glandular tissue—that were found in the wild-type tumours. Intriguingly, when induced in culture systems, the *Ugcg*^{-/-} embryonic stem cells were able to differentiate along erythroid and neuronal lineages.

These results demonstrate that glycosphingolipid synthesis was needed for embryonic development to proceed past the point of gastrulation. An essential function of glycosphingolipids in tissue differentiation was also documented by the absence of mature tissues in *Ugcg*^{-/-}

teratomas. In contrast to the *in vivo* results, significant differentiation was found to proceed in cell culture in the absence of glycosphingolipid synthesis, perhaps indicating the limitations of *in vitro* systems in determining these functions of glycosphingolipids (Kolter *et al.* 2000). Collectively, the results demonstrate the essential role of glycosphingolipid synthesis in embryonic development and cellular differentiation. A more detailed analysis awaits the ability to delete the *Ugcg* gene at particular times during development and in selected tissues.

4. β -1,4-*N*-ACETYL GALACTOSAMINYL-TRANSFERASE

The synthesis of the complex gangliosides prominent in the brain requires the activity of β -1,4-*N*-acetylgalactosaminyltransferase encoded by the *GalNAcT* gene (figure 1) (Sandhoff & Kolter 2003). Mice homozygous for a disrupted *GalNAcT* gene were viable, had a normal lifespan and initially appeared to be without significant neurological abnormalities (Takamiya *et al.* 1996; Liu *et al.* 1999). These mice were indeed deficient in complex gangliosides and expressed predominantly simple gangliosides, GM3 and GD3, in brain. Upon closer examination it was found that the mutant mice did in fact exhibit significant neurological abnormalities including impaired nerve conduction, Wallerian degeneration and age-related motor function defects (Takamiya *et al.* 1996; Sheikh *et al.* 1999; Chiavegatto *et al.* 2000). Impairment of the interaction between gangliosides and MAG may underlie some of these defects. MAG is a constituent of the myelin membrane where it binds the neuronal surface to promote axon-myelin stability (Frutiger *et al.* 1995). MAG is also believed to provide an inhibitory signal to neurons that prevents nerve regeneration (McKerracher *et al.* 1994; Mukhopadhyay *et al.* 1994; Schafer *et al.* 1996). MAG is a member of the Siglec (sialic acid-dependent immunoglobulin-like family member lectins) group and binds α 2-3 linked sialic acids carried by brain gangliosides (Yang *et al.* 1996; Collins *et al.* 1997, 1999). The *GalNAcT*^{-/-}

Table 1. Mice with disrupted glycosphingolipid synthesis genes.
(In wild-type mice the major brain gangliosides are GM1, GD1a, GD1b and GT1b.)

gene disrupted	major brain gangliosides	phenotype
<i>Ugcg</i>	N/A	lethal at gastrulation ^a
<i>GalNAcT</i>	GM3 and GD3	viable ^{b,c} ; impaired nerve conductance ^c ; motor function defects ^d ; impaired Ca ²⁺ regulation in neurons ^c ; dysfunctional interleukin-2 signalling in T cells ^f ; male infertility ^{b,g}
<i>GD3S</i>	GM1 and GD1a	viable ^{h,i} ; impaired regeneration of the hypoglossal nerve after lesioning ⁱ
<i>GalNAcT</i> and <i>GD3S</i>	GM3	viable with spontaneous adult lethal phenotype ^{h,j} ; sensitivity to audiogenic seizures ^h ; refractory skin lesion due to peripheral nerve degeneration ⁱ

^a Yamashita *et al.* (1999); ^b Liu *et al.* (1999); ^c Takamiya *et al.* (1996); ^d Chiavegatto *et al.* (2000); ^e Wu *et al.* (2001); ^f Zhao *et al.* (1999); ^g Takamiya *et al.* (1998); ^h Kawai *et al.* (2001); ⁱ Okada *et al.* (2002); ^j Inoue *et al.* (2002).

mice, which lack all complex gangliosides, are phenotypically similar to the *MAG*^{−/−} mice (Fruttiger *et al.* 1995; Yin *et al.* 1998). Both show dysmyelination and progressive neurodegeneration in the central and peripheral nervous system, suggesting a functional relationship between *MAG* and complex gangliosides (Sheikh *et al.* 1999).

Impaired Ca²⁺ regulation in neurons (Wu *et al.* 2001), dysfunctional interleukin-2 signalling in T cells (Zhao *et al.* 1999) and male infertility (Takamiya *et al.* 1998; Liu *et al.* 1999) have also been described in the *GalNAcT*^{−/−} mice.

The viability of *GalNAcT*^{−/−} mice and lack of apparent neurological abnormalities was initially surprising because of an expectation that complex gangliosides would have an essential role in the development of the mammalian nervous system. While subtle developmental abnormalities have not been ruled out, these results demonstrate that a nervous system can be formed without most complex gangliosides. However, it is now clear that the absence of complex gangliosides results in significant nervous system abnormalities.

5. GD3 SYNTHASE

The gene encoding GD3 synthase (*GD3S*) controls the synthesis of b-gangliosides (figure 1; Sandhoff & Kolter 2003). The *GD3S*^{−/−} mice were viable, fertile and without apparent neurological abnormalities (Kawai *et al.* 2001; Okada *et al.* 2002). In brain, the mutant mice lacked b-series gangliosides GD1b and GT1b. The a-series gangliosides GM1 and GD1a were present. These mice did exhibit impaired regeneration of the hypoglossal nerve after lesioning (Okada *et al.* 2002).

In view of the results obtained with the *GalNAcT*^{−/−} mice, the lack of a severe phenotype in the *GD3S*^{−/−} mice was perhaps not surprising. Although b-series gangliosides were absent, other complex ganglioside from the

a-series remained that may functionally compensate for their loss.

6. DOUBLE MUTANT MICE

The *GalNAcT* and *GD3S* mutant mice were crossbred to produce double mutant mice by two groups of investigators (Kawai *et al.* 2001; Inoue *et al.* 2002). Again, viable mice were obtained. As predicted by the biosynthetic pathway (figure 1), these double mutant mice expressed only GM3 ganglioside in brain. In contrast to either single mutant, the double mutant showed a very severe phenotype-spontaneous adult lethality. The double mutant mice began to die soon after weaning. By 30 weeks of age, over 50% of the mice had died. In one of the studies, the double mutant mice displayed exquisite sensitivity to sound-induced seizures (Kawai *et al.* 2001). Nearly 100% of the double mutant mice could be induced to have lethal seizures by jangling a standard set of house keys. This suggested that the adult lethality might be the result of seizures. However, the second independently derived line of the double knockout mice, which displayed the adult lethal phenotype, did not show susceptibility to audiogenic seizures, suggesting possible modifier background loci (Inoue *et al.* 2002). This second line of mice showed a propensity for skin injury. This phenotype may be attributable to an observed peripheral nerve degeneration leading to reduced sensory function and inadvertent injury to the skin.

The phenotype of double mutant mice illustrates the fundamental importance of gangliosides in nervous system function. The much milder phenotypes of the single mutants suggest that some ganglioside structures may be functionally redundant.

7. CONCLUSIONS AND PROSPECTS

The ability to delete groups of glycosphingolipid structures in mice has been tremendously important in developing an understanding of their physiological roles

(table 1). It is now certain that glycosphingolipid synthesis is required for the very early stages of embryonic development, although their precise functions at this stage remain to be determined. Elimination of complex ganglioside structures has revealed that most are expendable for the development of the mammalian nervous system. However, it is clear that there is an absolute requirement for these structures to produce proper nervous system function. In part, this is the result of a critical role for gangliosides in myelin stability and axonal-glia signalling. Other important nervous functions for gangliosides remain to be uncovered. Ultimately, identification of these functions may require analysis of mice with a total absence of all gangliosides.

Glycosphingolipids also have important functions outside the nervous system as indicated by defects in the reproductive and immune systems in mice lacking gangliosides. To tease out these functions, tissue-specific ablation of glycosphingolipid synthesis would be of great value. The determination of the normal functions of glycosphingolipids will eventually enable an understanding of how these lipids participate in pathological states such as in neurodegenerative disorders, cancer, and in diseases of metabolism and immune function.

REFERENCES

- Chiavegatto, S., Sun, J., Nelson, R. J. & Schnaar, R. L. 2000 A functional role for complex gangliosides: motor deficits in GM2/GD2 synthase knockout mice. *Exp. Neurol.* **166**, 227–234.
- Collins, B. E., Yang, L. J., Mukhopadhyay, G., Filbin, M. T., Kiso, M., Hasegawa, A. & Schnaar, R. L. 1997 Sialic acid specificity of myelin-associated glycoprotein binding. *J. Biol. Chem.* **272**, 1248–1255.
- Collins, B. E., Ito, H., Sawada, N., Ishida, H., Kiso, M. & Schnaar, R. L. 1999 Enhanced binding of the neural siglecs, myelin-associated glycoprotein and Schwann cell myelin protein, to Chol-1 (alpha-series) gangliosides and novel sulfated Chol-1 analogs. *J. Biol. Chem.* **274**, 37 637–37 643.
- Fruttiger, M., Montag, D., Schachner, M. & Martini, R. 1995 Crucial role for the myelin-associated glycoprotein in the maintenance of axon-myelin integrity. *Eur. J. Neurosci.* **7**, 511–515.
- Hakomori, S. I. 2002 Inaugural article: the glycosynapse. *Proc. Natl Acad. Sci. USA* **99**, 225–232.
- Harder, T. 2003 Formation of functional cell membrane domains: the interplay of lipid- and protein-mediated interactions. *Phil. Trans. R. Soc. Lond. B* **358**, 863–868. (DOI 10.1098/rstb.2003.1274.)
- Inoue, M., Fujii, Y., Furukawa, K., Okada, M., Okumura, K., Hayakawa, T. & Sugiura, Y. 2002 Refractory skin injury in the complex knock-out mice expressing only GM3 ganglioside. *J. Biol. Chem.* **277**, 22.
- Kasahara, K., Watanabe, Y., Yamamoto, T. & Sanai, Y. 1997 Association of Src family tyrosine kinase Lyn with ganglioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolipid in caveolae-like domains. *J. Biol. Chem.* **272**, 29 947–29 953.
- Kasahara, K., Watanabe, K., Takeuchi, K., Kaneko, H., Oohira, A., Yamamoto, T. & Sanai, Y. 2000 Involvement of gangliosides in glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule TAG-1 signaling in lipid rafts. *J. Biol. Chem.* **275**, 34 701–34 709.
- Kawai, H. (and 11 others) 2001 Mice expressing only monosialoganglioside GM3 exhibit lethal audiogenic seizures. *J. Biol. Chem.* **276**, 6885–6888.
- Kolter, T., Magin, T. M. & Sandhoff, K. 2000 Biomolecule function: no reliable prediction from cell culture. *Traffic* **1**, 803–804.
- Kolter, T., Proia, R. L. & Sandhoff, K. 2002 Combinatorial ganglioside biosynthesis. *J. Biol. Chem.* **277**, 25 859–25 862.
- Liu, Y. (and 12 others) 1999 A genetic model of substrate deprivation therapy for a glycosphingolipid storage disorder. *J. Clin. Invest.* **103**, 497–505.
- McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J. & Braun, P. E. 1994 Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* **13**, 805–811.
- Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R. & Filbin, M. T. 1994 A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* **13**, 757–767.
- Okada, M. (and 12 others) 2002 b-series Ganglioside deficiency exhibits no definite changes in the neurogenesis and the sensitivity to Fas-mediated apoptosis but impairs regeneration of the lesioned hypoglossal nerve. *J. Biol. Chem.* **277**, 1633–1636.
- Prinetti, A., Prioni, S., Chigorno, V., Karagogeos, D., Tettamanti, G. & Sonnino, S. 2001 Immunoseparation of sphingolipid-enriched membrane domains enriched in Src family protein tyrosine kinases and in the neuronal adhesion molecule TAG-1 by anti-GD3 ganglioside monoclonal antibody. *J. Neurochem.* **78**, 1162–1167.
- Sandhoff, K. & Kolter, T. 2003 Biosynthesis and degradation of mammalian glycosphingolipids. *Phil. Trans. R. Soc. Lond. B* **358**, 847–861. (DOI 10.1098/rstb.2003.1265.)
- Schafer, M., Fruttiger, M., Montag, D., Schachner, M. & Martini, R. 1996 Disruption of the gene for the myelin-associated glycoprotein improves axonal regrowth along myelin in C57BL/Wlds mice. *Neuron* **16**, 1107–1113.
- Sheikh, K. A., Sun, J., Liu, Y., Kawai, H., Crawford, T. O., Proia, R. L., Griffin, J. W. & Schnaar, R. L. 1999 Mice lacking complex gangliosides develop Wallerian degeneration and myelination defects. *Proc. Natl Acad. Sci. USA* **96**, 7532–7537.
- Simons, K. & Toomre, D. 2000 Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39.
- Simons, M., Friedrichson, T., Schulz, J. B., Pitto, M., Masserini, M. & Kurzchalia, T. V. 1999 Exogenous administration of gangliosides displaces GPI-anchored proteins from lipid microdomains in living cells. *Mol. Biol. Cell* **10**, 3187–3196.
- Takamiya, K. (and 13 others) 1996 Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system. *Proc. Natl Acad. Sci. USA* **93**, 10 662–10 667.
- Takamiya, K. (and 11 others) 1998 Complex gangliosides are essential in spermatogenesis of mice: possible roles in the transport of testosterone. *Proc. Natl Acad. Sci. USA* **95**, 12 147–12 152.
- Wu, G., Xie, X., Lu, Z. H. & Ledeen, R. W. 2001 Cerebellar neurons lacking complex gangliosides degenerate in the presence of depolarizing levels of potassium. *Proc. Natl Acad. Sci. USA* **98**, 307–312.
- Yamashita, T., Wada, R., Sasaki, T., Deng, C., Bierfreund, U., Sandhoff, K. & Proia, R. L. 1999 A vital role for glycosphingolipid synthesis during development and differentiation. *Proc. Natl Acad. Sci. USA* **96**, 9142–9147.
- Yang, L. J., Zeller, C. B., Shaper, N. L., Kiso, M., Hasegawa, A., Shapiro, R. E. & Schnaar, R. L. 1996 Gangliosides are neuronal ligands for myelin-associated glycoprotein. *Proc. Natl Acad. Sci. USA* **93**, 814–818.

Yin, X., Crawford, T. O., Griffin, J. W., Tu, P., Lee, V. M., Li, C., Roder, J. & Trapp, B. D. 1998 Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. *J. Neurosci.* **18**, 1953–1962.

Zhao, J., Furukawa, K., Fukumoto, S., Okada, M., Furugen, R., Miyazaki, H., Takamiya, K., Aizawa, S., Shiku, H. & Matsuyama, T. 1999 Attenuation of interleukin 2 signal in

the spleen cells of complex ganglioside-lacking mice. *J. Biol. Chem.* **274**, 13 744–13 747.

GLOSSARY

GPI: glycosylphosphatidylinositol

MAG: myelin-associated glycoprotein