Chapter 14
Glycosphingolipids in the Regulation of the Nervous System

Koichi Furukawa, Yusuke Ohmi, Yuki Ohkawa, Orie Tajima, and Keiko Furukawa

Abstract The highest expression of gangliosides, sialic acid-containing glycosphingolipids (GSLs), is found in the nervous tissue of vertebrates. Changes in the profiles of gangliosides during the development of nervous tissues indicate that they are involved in the regulation of neurogenesis and synaptogenesis. Their distinct distribution patterns support the suggestion that they are involved in both the differentiation and function of neural cells. In addition to results of studies of GSLs done using biochemical, histopathological, and cell biological approaches, recent progress in the genetic engineering of glycosyltransferase genes has resulted in novel findings and concepts about their roles in the nervous system. Roles of GSLs in the regulation of signaling that determine cell fates in membrane microdomains such as lipid rafts have been extensively studied. In particular, gene targeting of glycosyltransferases in mice has enabled investigation of the in vivo functions of GSLs. The majority of abnormal phenotypes exhibited by knockout (KO) mice may reflect an abnormal structure and a resultant altered function of lipid rafts caused by alterations in their GSL composition. Generally speaking, abnormal phenotypes found in most KO mice were milder than expected, suggesting that the remaining GSLs compensate for the functions of those lost. There are also functions that

K. Furukawa (✉) • Y. Ohmi • Y. Ohkawa
Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan
e-mail: koichi@med.nagoya-u.ac.jp

O. Tajima
Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai 487-8501, Japan

K. Furukawa
Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan

Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai 487-8501, Japan

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cannot be replaced by the remaining GSLs. Thus, there may be two modes of function of GSLs: one is nonspecific and can be carried out by multiple GSLs, the second mode is that in which the function of the missing GSL(s) cannot be compensated by others. Identification of natural ligands for individual GSLs is crucial in order to clarify the functions of each structure.

Keywords Glycosphingolipids • Microdomains • Gangliosides • Knockout • NGF • Lipid rafts

Abbreviations

GSLs	Glycosphingolipids
KO	Knockout
GlcCer	Glucosylceramide
LacCer	Lactosylceramide
GalCer	Galactosylceramide
DKO	Double KO
NGF	Nerve growth factor
CNS	Central nervous system
GPI	Glycosylphosphatidylinositol.

14.1 Introduction

Glycosphingolipids (GSLs) are unique amphipathic molecules consisting of a hydrophilic carbohydrate moiety and a hydrophobic lipid portion (Wiegandt 1985) majority of which is synthesized from glucosylceramide (GlcCer). The carbohydrate moiety can usually be classified into one of 4 major series. They are the ganglio-, globo-, lacto/neolacto-, and asialo-series. Lactosylceramide (LacCer), a common precursor of most GSLs is synthesized by addition of galactose (in a β1,4-linkage) to GlcCer. In addition, gala-series GSLs generated from galactosylceramide (GalCer) are also present. The GalCer-derived GSLs is limited in number as are their sites of expression. In addition to polymorphism in the carbohydrate moiety of GSLs, variability is seen in both their fatty acid and long-chain base composition. Thus, regulatory systems needed for proper expression of GSLs should be present in tissues in which they are found as well as at specific stages of cell/tissue differentiation.

Histopathological and biochemical studies of cells maintained in vitro and of various organs and tissues resulted in the identification of a number of biological functions for individual GSLs (Schengrund 1990; Yu et al. 1988). In particular,

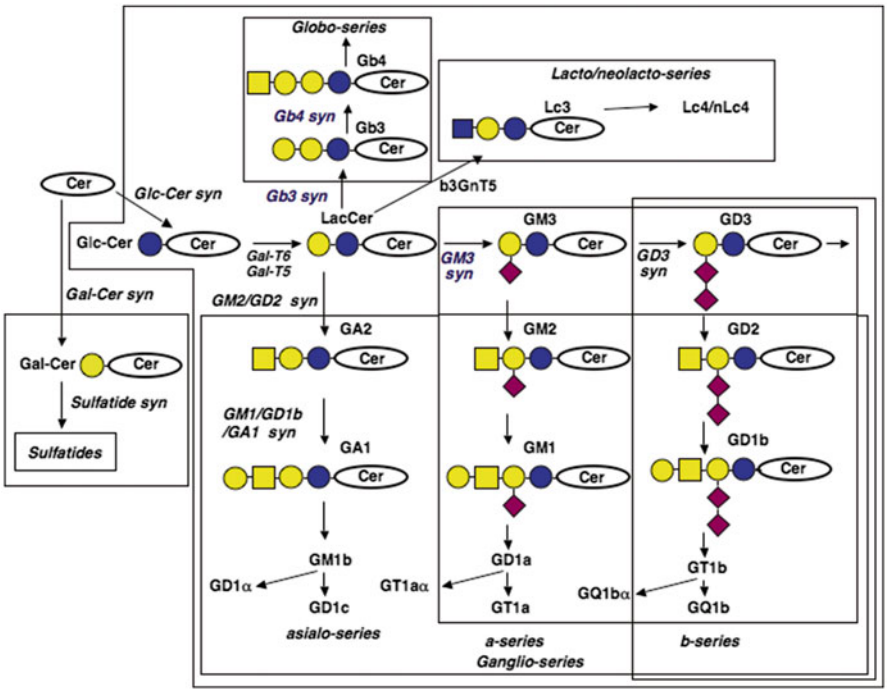

Fig. 14.1 Synthetic pathway and enzymes needed for GSL synthesis. The synthetic pathway and main enzymes responsible for GSL synthesis are shown. Deleted structures in individual knockout mice are indicated by *squares*

interrogation of neural cell lines sensitive to well-defined differentiation factors resulted in novel findings regarding mechanisms by which GSLs may affect cell function (Greene and Tischler 1976; Levi et al. 1988; Mutoh et al. 1995). Since the isolation of cDNA encoding the glycosyltransferase responsible for synthesis of GM2 and GD2 (Nagata et al. 1992), a number of other glycosyltransferase cDNAs have been isolated (Lloyd and Furukawa 1998). The synthetic pathways and enzymes for GSL synthesis are shown in Fig. 14.1. Isolation of the specific cDNAs facilitated studies of knockout (KO) animals, in which specific glycosyltransferase genes were genetically disrupted (Furukawa et al. 2001). Functions of missing GSLs were identified based on the phenotypes seen in the various glycosyltransferase gene KO mice (Furukawa et al. 2001). However, KO of specific glycosyltransferase genes in mice often resulted in milder abnormal phenotypes than expected (Furukawa et al. 2001, 2004).

In this chapter we summarize the roles of GSLs in the nervous system as identified using cultured cells and mice in which specific GSL synthase genes were knocked out, and discuss anticipated future research.

14.2 Glycosphingolipids in Cultured Cells

14.2.1 Gangliosides Modulate Signals Transduced by Neurotrophic Factors/Receptors

Gangliosides are highly expressed in nervous tissue, and have been considered to be involved in the regulation of their development, differentiation, and function (Schengrund 1990). A number of studies have reported that gangliosides play roles as neurotrophic factors in cultured neural cell lines and anti-apoptotic factors (Ferrari et al. 1983). Studies of a rat pheochromocytoma cell line PC12 demonstrated that gangliosides regulate proliferation (Fukumoto et al. 2000) and differentiation (Nishio et al. 2004) of cells by modulating cell signaling. Nerve growth factor (NGF) binds TrkA and triggers phosphorylation of the receptor and subsequent activation of Ras/Raf/MEK/Erk pathway leading to neurite extention (Vaudry et al. 2002). To our surprise, over-expression of ST8SIA1 (GD3 synthase) cDNA resulted in the constitutive activation of TrkA and Erk1/2, and consequently unresponsiveness to NGF. These cells showed continuous cell growth even after NGF treatment. GD3 synthase cDNA transfectant cells may represent the features of neural cells during early brain development, a time period in which GD3 is a dominant ganglioside (Yu et al. 1988). On the other hand, over-expression of GM1 synthase induced unresponsiveness to nerve growth factor (NGF). In this case, no phosphorylation of TrkA and subsequent signaling molecules could be found even after NGF treatment. In these transfectant cells, a dramatic alteration in the intracellular localization (inside to outside of lipid rafts) of the NGF receptor TrkA and other signaling molecules such as $p75^{NTR}$ and H-Ras was observed. Results of earlier studies had indicated that GM1 could act as a neurotrophic factor in the rescue of serum-deprived PC12 cells (Ferrari et al. 1983) as well as in the enhancement of neurite extension (Mutoh et al. 1995). Although the reason for the difference in the effects of GM1 (exogenous vs endogenous) on PC12 cells are not known, relative GM1 levels might be critical for the direction of cell signaling. The aberrant signaling seen in transfected cells is shown in Fig. 14.2. These observations are examples of the roles of lipid raft-associated gangliosides in the regulation of signal transduction.

14.2.2 Essential Roles of GSLs for Development of Multicellular Organisms

As shown by a UGCG (GlcCer synthase)-deficient cell line (Ichikawa et al. 1996), GSLs are not essential for the growth and survival of mammalian cells in culture. On the other hand, in GlcCer synthase-KO mice GSLs were needed for development at embyonic day E6.5–7.5. The GlcCer synthase-deficient mutant mice showed embryonal lethality by E7.5 accompanied by intensive apoptotic changes particularly in the ectoderm. This observation indicated that GSLs are essential for

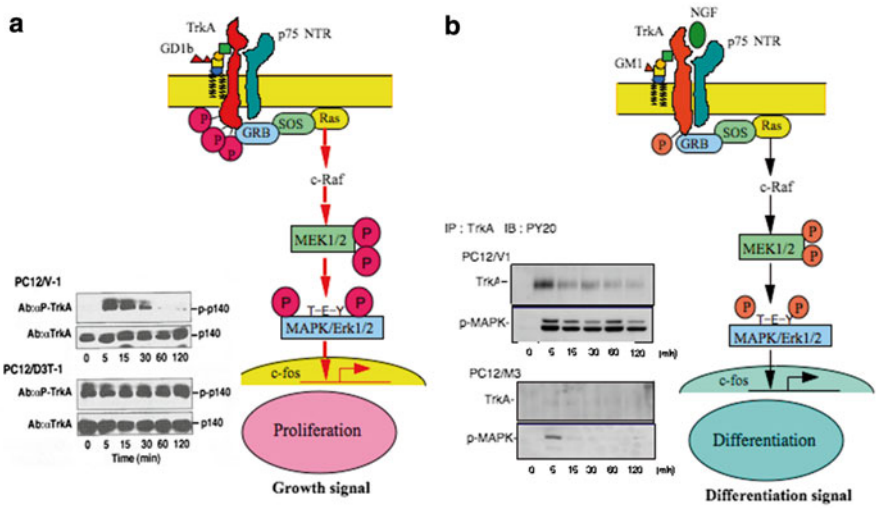

Fig. 14.2 Aberrant signaling seen in cells transfected with specific glycosyltransferase cDNAs. (**a**) Transfection of GD3 synthase cDNA into a PC12 cell line resulted in constitutive activation of TrkA and ERK1/2, which was accompanied by enhanced cell proliferation and unresponsiveness to NGF. (**b**) Transfection of PC12 cells with B3galt4 (GM1/GD1b synthase) cDNA resulted in the cells being unresponsive to NGF stimulation both in neurite extension and in TrkA/Erk1/2 activation

development of multicellular organisms (Yamashita et al. 1999). Conditional knockout (KO) of GlcCer synthase in mice, in which the gene was destroyed after birth, resulted in severe dysfunction of cerebellum and peripheral nerves that was associated with structural defects (Jennemann et al. 2005). The fact that the mice died within 3 weeks after birth again indicated that GSLs are indispensable for the maintenance and survival of organisms.

14.3 Impact of KO of Glycosyltransferase Genes Located at Stem Steps of GSL Synthesis

14.3.1 KO of ST3GAL5 (GM3 Synthase) in Mice

GM3 synthase is essential for the synthesis of GM3 from LacCer, and therefore, for the synthesis of all ganglio-series gangliosides. However, KO of the gene in mice resulted in no apparent abnormal phenotype except increased sensitivity to insulin (Yamashita et al. 2003). This phenotype is in dramatic contrast to that seen in patients with GM3 synthase deficiency (Simpson et al. 2004). Patients with the mutated GM3 synthase gene exhibit "infantile-onset symptomatic epilepsy" with growth and mental retardation. The basis for this marked difference between human and mouse is not known.

14.3.2 KO of UGT8 (GalCer Synthase) and GAL3ST1 (Sulfatide Synthase) in Mice

GalCer-derived GSLs were shown in studies of mutant mice lacking the GalCer synthase gene (Coetzee et al. 1996) to generate myelin that contained glucocerebroside. In addition to lacking GalCer these mice also lacked sulfatide (another myelin-enriched GSL) and seminolipid. The fact that these mice had severe neurological defects (Coetzee et al. 1996) indicated that GalCer and/or sulfatide has an important role in myelin. The mutants also showed male sterility (Fujimoto et al. 2000).

Sulfatide synthase is essential for synthesis of both sulfatides and seminolipids, neither of which was expressed in sulfatide synthase KO mice (Honke et al. 2002). Sulfatides are expressed mainly in oligodendrocytes in the CNS and in Schwann cells in peripheral nerves. As expected the sulfatide synthase KO mice had phenotypes similar to those of GalCer synthase-disrupted mice, but the abnormalities were milder (Honke et al. 2002).

14.3.3 LacCer Synthase KO Mice

B4galt6 (Lactosylceramide synthase, LacCer synthase) KO mice showed no definite abnormal phenotypes (Tokuda et al. 2013). In contrast, KO of B4galt5 resulted in severe defects in development, indicating that β1,4Gal-T5 might be the main LacCer synthase (Kumagai et al. 2009; Nishie et al. 2010). The phenotype resulting from disruption of the LacCer synthase was similar to that induced by KO of GlcCer synthase.

14.4 Compensation for Lost Functions by Remaining GSLS

Generally, disruption of ganglioside synthases resulted in milder phenotypes than expected. In particular, the fact that KO mice lacking the B4galnt1 (GM2/GD2 synthase) gene had an almost normal architecture of the central nervous system (CNS) at birth despite the lack of all complex gangliosides was quite surprising (Takamiya et al. 1996). No clear differences between KO and wild-type mice were found in brain morphology, myelination, and behavior. The only change observed was a reduction in nerve conductivity. Male infertility due to aspermatogenesis appeared to be the most serious phenotype (Takamiya et al. 1998). However, KO mice underwent neuronal degeneration that increased gradually with aging (Sugiura et al. 2005). The degenerative disorders were detected primarily in peripheral nerves and the dorsal horn of the spinal cord (Sugiura et al. 2005). These KO mice showed sensory nerve-dominant neurodegeneration, while another group using the same kind of KO mice reported Wallerian degeneration and abnormal neurological

function in a motor-neuron-dominant manner (Sheikh et al. 1999; Chiavegatto et al. 2000). Morphological changes in synaptic vesicles and dendrites in the central terminals, and in glia processes indicated that compensatory modification of neural tissue took place after nerve degeneration, i.e., remodeling or regeneration. Furthermore, regeneration of resected hypoglossal nerves was strongly disturbed (Kittaka et al. 2008). These results indicated that complex gangliosides are not essential in morphogenesis, but important in the maintenance and repair of nerve tissue. Aberrant Ca^{2+}-regulating properties in the cerebellar neurons found in the same type of KO mice (Wu et al. 2001) may correspond with the neurological disorders described above. All these results indicate that increased GM3 and GD3 play important roles in compensating for the loss of more complex gangliosides as seen in GM2/GD2 synthase KO mice.

Despite the fact that genetic disruption of the GD3 synthase gene resulted in loss of all b-series and c-series gangliosides (Kawai et al. 1998; Okada et al. 2002), almost no apparent abnormalities were seen in either morphology or behavior. Although GD3 was reported to mediate the apoptotic signals mediated by Fas–Fas ligands (De Maria et al. 1997), sensitivity of thymocytes from these KO mice to apoptosis induced by anti-Fas antibody appeared unaffected. While no morphological abnormalities were detected in neural tissue of GD3 synthase KO mice, significantly reduced regenerative activity was found in hypoglossal nerve resection experiments (Okada et al. 2002). This indicated that b-series gangliosides have a crucial role in nerve regeneration, and supported observations indicating that b-series gangliosides were the most effective at stimulating rat hypoglossal nerve regeneration (Itoh et al. 2001).

14.5 Double KO Exhibited More Severe Phenotypes

As summarized in Fig. 14.3, profiles of GSL species in the individual KO mice support the hypothesis that remaining ones might compensate for the roles of lost GSLs. This could be due to two important possibilities. The first is that the total cell concentration of GSLs is strictly regulated so that their total concentration is similar despite variability in their composition. The second is that functions of some GSLs can be replaced by others. In order to evaluate the roles of each GSL we generated complex KO mice in which remaining GSLs were reduced as much as possible.

In double KO (DKO) mice lacking both GD3 synthase and GM2/GD2 synthase genes, animals only synthesized GM3 (Inoue et al. 2002), no obvious changes were detected at birth. However, they gradually died of unknown causes about 12 weeks after birth. They also exhibited refractory skin lesions on the face and neck. Reduced sensitivity to mechanical pain seemed to trigger the skin lesions. Neurodegenerative changes at a fairly young age may explain the dysfunction in the sensory system (Inoue et al. 2002). Audiogenic seizures and consequent sudden death by noise were observed in another group of the double DKO mice (Kawai et al. 2001). Interestingly, our DKO mice did not show the same response to noise. This difference

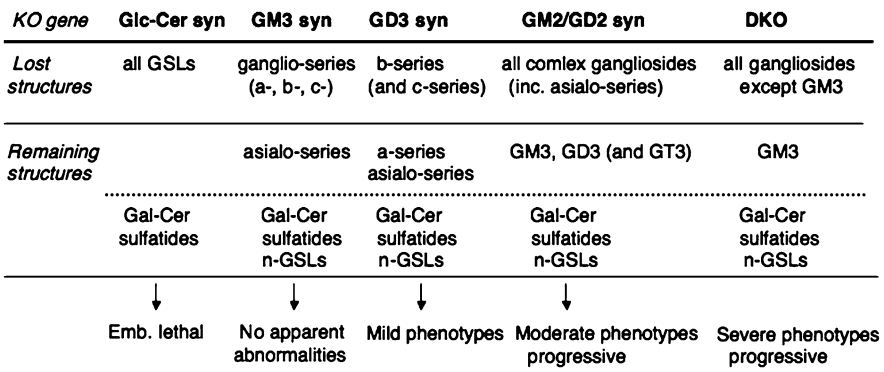

Fig. 14.3 Profiles of GSL species synthesized by various KO mice. GSLs found in the KO mice lines are shown. These structures may compensate for the role(s) of GSLs missing in the individual KO mice. DKO, double knockout of GD3 synthase and GM2/GD2 synthase genes. n-*GSLs* neutral glycosphingolipids, *Emb. Lethal* embyonal lethal

may be due to the different genetic backgrounds of the ES lines used. Phenotypic analyses of these mutant mice indicated that GM3 alone enabled them to undergo almost normal neurogenesis, birth, and development up to a certain point. However, the correct composition of gangliosides seemed to be essential for maintenance of intact morphology and function. Subsequent analyses to clarify the cause of neurodegeneration seen in the DKO mice revealed that in order to maintain integrity in the architecture and function of nervous tissue cells lipid rafts containing the appropriate composition of GSLs were needed (Ohmi et al. 2009, 2011).

Mice in which both GM3 synthase and GM2/GD2 synthase genes were knocked out were also generated (Yamashita et al. 2005) and used to examine the significance of asialo-series gangliosides. These animals eventually showed severe nerve degeneration, leading to an early death. The severity of this phenotype indicates that GM3 itself is essential for survival as well as maintenance of the CNS. Although GM3 has been reported to suppress EGF/EGFR-mediated signals by forming complexes with tetraspanin (Yoon et al. 2006), the specific effect of its loss in the DKO mice remains to be further clarified.

14.6 Response to Neurodegeneration by Modification of Gene Expression in the DKO mice

Changes in gene expression profiles in DKO mice were examined using DNA microarrays. The results indicated that an up- (or down-) regulation in expression of genes encoding proteins involved in inflammation- and immunological reaction-related events, and of those encoding proteins that react to inflammation and/or degeneration took place in KO brains. This grouping scheme is shown in Fig. 14.4. Up-regulation of expression of genes encoding proteins such as complement C4 and

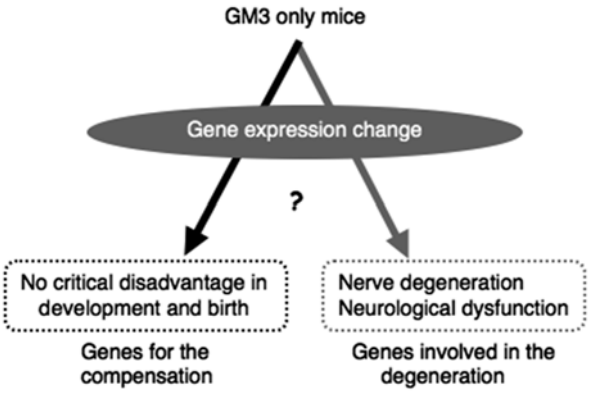

Fig. 14.4 Genes strongly upregulated or downregulated in nervous tissue of DKO mice. cDNA microarray analyses were performed to compare gene expression profiles between wild-type and DKO mouse brain tissue. One group seemed to include inflammation- and immunological reaction-related genes. The other group contained those regulated as a reaction to inflammation and/or degeneration in DKO brains. The former might be results of defects of gangliosides, and the latter might represent host reaction to inflammation and/or degeneration caused by ganglioside deficiency

C3a receptor 1, indicated that inflammation could be a major cause of the neurodegeneration seen. Support for this hypothesis was provided by results obtained in studies of triple KO mice lacking GM2/GD2 synthase, GD3 synthase, and C3 (Ohmi et al. 2009).

An increased expression of genes encoding anti-apoptotic proteins was also observed (Ohmi et al. 2011). Among upregulated genes in cerebellum of the DKO mice, was Wisp2/CCN5. This was of interest because expression of this gene in the CNS had not been previously reported. Its over-expression resulted in increased cell proliferation and neurite outgrowth upon serum withdrawal from cultured Neuro2a cells (Ohkawa et al. 2011). Integrin appears to be a receptor for secreted Wisp2/CCN5. The cDNA-transfected cells also exhibited resistance to H_2O_2-induced apoptosis. These results indicate that the Wisp2/CCN5 induced in neurons of DKO mice serves to protect them from neurodegeneration caused by ganglioside deficiency. Changes found in nervous tissue of DKO mice are summarized in Fig. 14.5.

14.7 Mechanisms by Which Gangliosides May Maintain the Integrity of the CNS

DKO of two major glycosyltransferase genes showed more severe neurodegeneration than those detected in single KO animals. Studies of potential mechanisms underlying neurodegeneration identified a role for gangliosides in regulation of the complement system (Inoue et al. 2002; Ohmi et al. 2009). Gangliosides in membrane microdomains were found to control the complement system and suppression of inflammation and neurodegeneration (Ohmi et al. 2012). Results of studies of

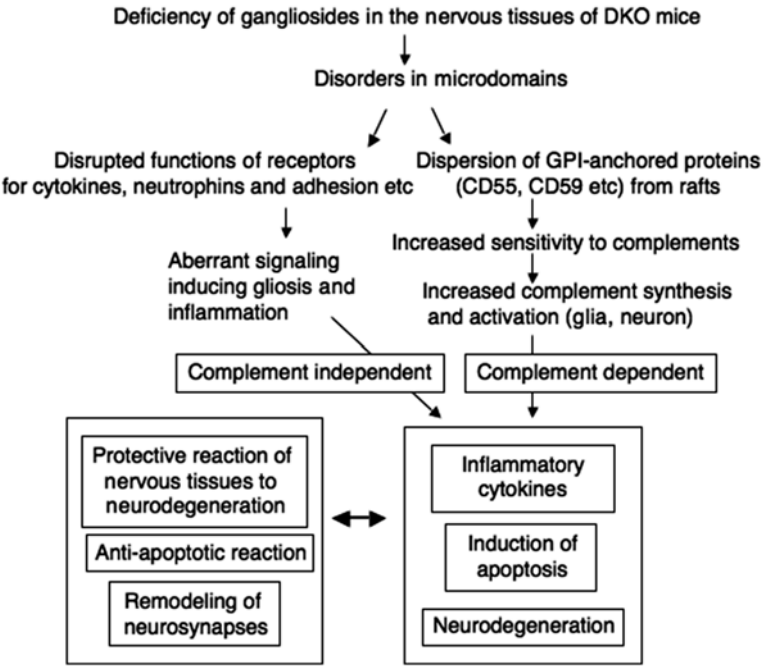

Fig. 14.5 Defects and reactions observed in nervous tissue of DKO mice

mice in which specific ganglioside synthases were knocked out indicated that the disruption of lipid rafts was accompanied by up-regulation of complement-related genes associated with proliferation of astrocytes and infiltration of microglia. Severity of the effect depended on the defects in ganglioside composition. Glycosylphosphatidylinositol (GPI)-anchored molecules such as DAF, CD59, and NCAM tended to disperse most severely from the raft fraction from DKO mice > GM2/GD2 synthase KO > GD3 synthase KO > WT. Even lipid raft markers such as flotillin-1 dispersed from the raft fractions in a similar order. These results indicated that the architecture of the lipid rafts was destroyed by deletion of gangliosides and that the degree of disruption depended on the severity of the change in GSL composition (Ohmi et al. 2011).

14.8 Potential Relationships Between the Role(s) of GSLS in the Regulation of the Architecture and Function of Lipid Rafts and Human Neurodegenerative Diseases

The inflammatory responses seen in nervous tissue in KO mice are similar to those seen in human neurodegenerative diseases such as Alzheimer and Parkinson's diseases (Rogers et al. 1992; Shen et al. 2001). Neurodegeneration in these diseases is

frequently associated with autoimmune reactions. As shown in the triple KO mice described previously, immune suppression such as deprivation of complement components has been tried as a therapeutic approach (Sardi et al. 2011; Shen and Meri 2003). In addition, administration of antibodies or vaccination with disease-related proteins often alleviates pathological and clinical features associated with these neurodegenerative diseases (Delrieu et al. 2012; Shah and Federoff 2011).

The majority of studies on lipid rafts have been performed using cultured cell lines (Patra 2008; Simons and Gerl 2010), and not by experimental animals (Furukawa et al. 2007). On the other hand, many findings described in this chapter were substantially proven by analyzing molecular reactions in/near lipid rafts in the individual experimental systems with some done using brain tissue as the source of microdomains (Ohmi et al. 2009, 2011, 2012; Furukawa et al. 2011). Thus, it is reasonable to conclude that the majority of abnormal phenotypes observed in KO mice are due to disruption of the architecture and function of lipid rafts due to altered expression of GSLs.

14.9 Conclusions/Future Directions

We propose that there are two ways by which GSLs in lipid rafts function in the maintenance of structure and function of nervous tissue. The first is to help maintain the fundamental environment of the cell membrane, and the second is for each GSL to serve a particular function(s) that cannot be replaced by other glycoconjugates.

The availability of glycosyltransferase genes and mutant mice lacking them has provided a powerful tool for studying their role(s) in biological processes, such as development, cell growth, differentiation, and cell death. Interestingly, many of the novel findings obtained by analysis of the mutant mice are more complicated than expected, indicating that GSLs may have multiple functions. While some functions of the gangliosides can be compensated for by other molecules, others seem to be indispensable. In particular, it was quite surprising that GM3 synthase gene knockout mice (Yamashita et al. 2003) had no apparent morphological or behavioral abnormalities, suggesting that asialo-series gangliosides may have compensated for the loss of all GM3-derived gangliosides. Use of mice genetically engineered to lack specific glycosyltransferases should help clarify the role(s) of functional redundancy among GSLs. In cases where a specific role of a particular GSL cannot be replaced by other species it is possible that their function depends upon the interaction of the GSL with a specific ligand(s). Therefore, identification of these ligands is needed in order to understand how each GSL functions.

Finally, the question of where gangliosides/GSLs are located on the membranes of living cells remains to be interrogated. It may be possible to address this question as well as the question of whether they are located in close juxtaposition of the proteins with which they putatively interact using single molecule imaging with high spatiotemporal resolution (Suzuki et al. 2012, 2013).

Conflict of Interest The authors declare that they have no conflict of interest.

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