REVIEW

# Regulatory Mechanisms of Nervous Systems with Glycosphingolipids

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**Abstract** A number of studies have suggested functions of sialic acid-containing glycosphingolipids (gangliosides) in the nervous system. However, results of analyses of the mutant mice lacking gangliosides suggested that they play crucial roles in the maintenance of integrity and repair of the nervous tissues. Furthermore, results of double knockout mice lacking all gangliosides except GM3 (GM3-only mice) suggested that deficiency of gangliosides induced complement activation and inflammation, leading to neurodegeneration. Generation of triple knockout mice by mating GM3-only mice and C3-deficient mice verified the involvement of complement systems in the inflammation and neurodegeneration. For the mechanisms of the complement activation, functional disorders of complement-regulatory proteins such as CD55 and CD59, which belong to GPI-anchored proteins, should be main factors. These results suggested that normal composition of gangliosides is essential for the maintenance of lipid rafts. Therefore, it was suggested that regulation of the complement systems and suppression of the inflammation should be important for the treatment of neurodegeneration, having common aspects with other neurodegenerative diseases such as Alzheimer disease.

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## Introduction

Although a number of studies on the involvement of carbohydrate chains in various biological processes have been reported, it is relatively recent that their molecular mechanisms are elucidated. Since remodeling of carbohydrate chains in complex carbohydrates expressed on cells became capable by genetic manipulation of sugar chain-synthetic enzymes, mechanisms for the regulation of bio-organisms including signal transduction during differentiation and development, proliferation and adhesion of cancer cells, and invasion of pathogenic microorganisms such as bacteria and viruses have been clarified by studies based on the interactions between complex carbohydrates and their binding molecules.

The nervous system is the tissue where sialic acid-containing glycosphingolipids, gangliosides, were detected for the first time [1], and gangliosides are markedly expressed at very high levels. Therefore, a number of trials to investigate roles and mechanisms of complex carbohydrates in its organogenesis and functions have been performed [2]. Nevertheless, no sufficient progress has been achieved.

In this review, we summarized findings reported to date about abnormal phenotypes of glycosylation-defective mutant mice including our own studies with focus on the implication of gangliosides in the organogenesis, maintenance of functions, repair after injury, and various neurological diseases. Then, approaches in the future to elucidate carbohydrate functions specifically regulated by individual structures were discussed.

## Specific Features of Ganglioside Functions Based on Their Structures

Glycosphingolipids are amphopathic molecules, and consist of hydrophobic ceramide portion embedded into the outer layer of lipid bi-layer membrane, and hydrophilic carbohydrate moiety exposing outside of the cell surface. The ligand molecules that interact with the carbohydrates on glycolipids need to access to the carbohydrate portions from outside of cells (*trans*-action), or from the same membrane where ligands and glycolipids coexist (*cis*-action) [3]. Therefore, structural variety in the ceramide portions affects affinity of glycolipids with cell membrane and binding with membrane-constituting lipids, regulating the intracellular localization and spatial orientation of glycolipids in the membrane. On the other hand, carbohydrate moiety should determine the binding specificity and affinity with their ligand molecules in the interactions. Consequently, polymorphic carbohydrate structures on glycolipids regulated by various intrinsic and extrinsic conditions such as development, differentiation, transformation, and environmental stresses might determine the specificity of interaction with their ligands. In turn, ceramide portions might regulate the appropriate localization of the individual molecules, leading to the reasonable regulation of whole molecules.

One of the most important aspects in the functions of glycolipids suggested by the past studies is that they play roles by interacting with various proteins expressed on the same membrane as a cis-action directly and indirectly. This fact makes it possible that glycolipids form molecular clusters with particular molecules with high affinity, and also they exclude some molecules from their clusters by generated repulsive force. Furthermore, it is possible that glycolipids play roles forming individual clusters on the cell membrane. Therefore, we need to pay attention to the mechanisms for the glycolipid actions via cluster formation among glycolipids and/or glycolipids and other molecules.

## Functions of Gangliosides Elucidated by Glycolipid-Deficient Mutant Mice

It has been long believed by many researchers [4] that glycosphingolipids play important roles in the brain and nervous system based on their high level expression and strict regulation of their expression in a spatio-temporal manner [5] in nervous tissues. They have been considered to be involved in the organogenesis and neural functions based on the results of various experiments in vitro [2]. These considerations need to be partly modified by the findings obtained from the analyses of glycolipids-deficient mutant mice, and need to be newly defined [6]. As shown in Fig. 1, we and other groups have established knockout mice of following glycosyltransferase genes, i.e. GM2/GD2 synthase, GD3 synthase, GlcCer synthase, GalCer synthase and GM3 synthase. We also established knockout mice of Gb3/CD77 synthase, a key enzyme for the synthesis of globo-series glycolipids, and knockout mice of LacCer synthase (β4GalT-VI), a glycosyltransferase for the synthesis of the precursor structure for almost all of higher glycolipids. Moreover, knockout mice of sulfatide synthase gene were also reported [7].

First of all, in the KO mice lacking GM2/GD2 synthase responsible for the synthesis of all complex gangliosides [8], they were born with no apparent morphological abnormalities and grew up with no marked disorders. Therefore, it was shown that complex gangliosides are not essential in the neurite extension and synapse formation during neural development. However, these mutant mice exhibited abnormal behaviors and pathological features as found in neurodegenerative diseases when observed for a



Fig. 1 Synthetic pathway of gangliosides and knockout of glycosyltransferases. Main synthetic pathway of gangliosides are shown. Ganglioside structures deleted in knockout mice of GM2/GD2 synthase (*dotted line*) and GD3 synthase (*dashed line*) genes were enclosed by *individual lines*. In double knockout mice, all gangliosides except for GM3 were deleted
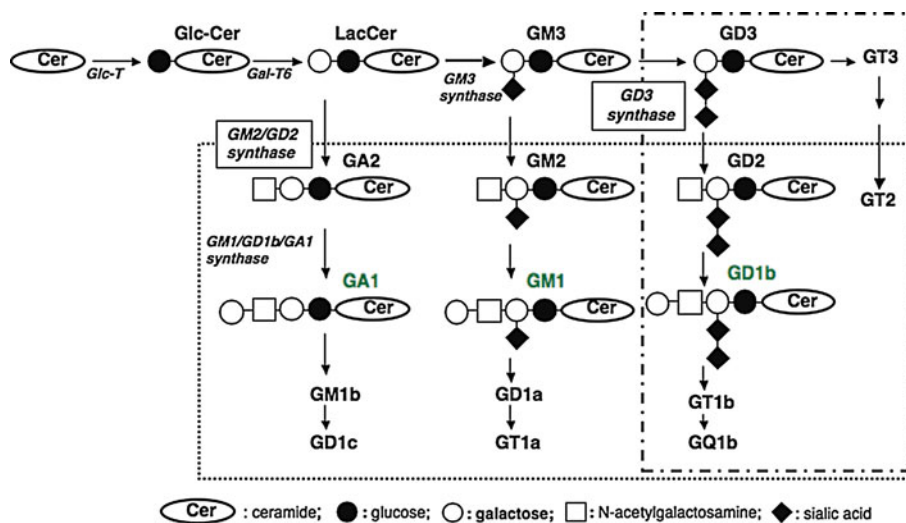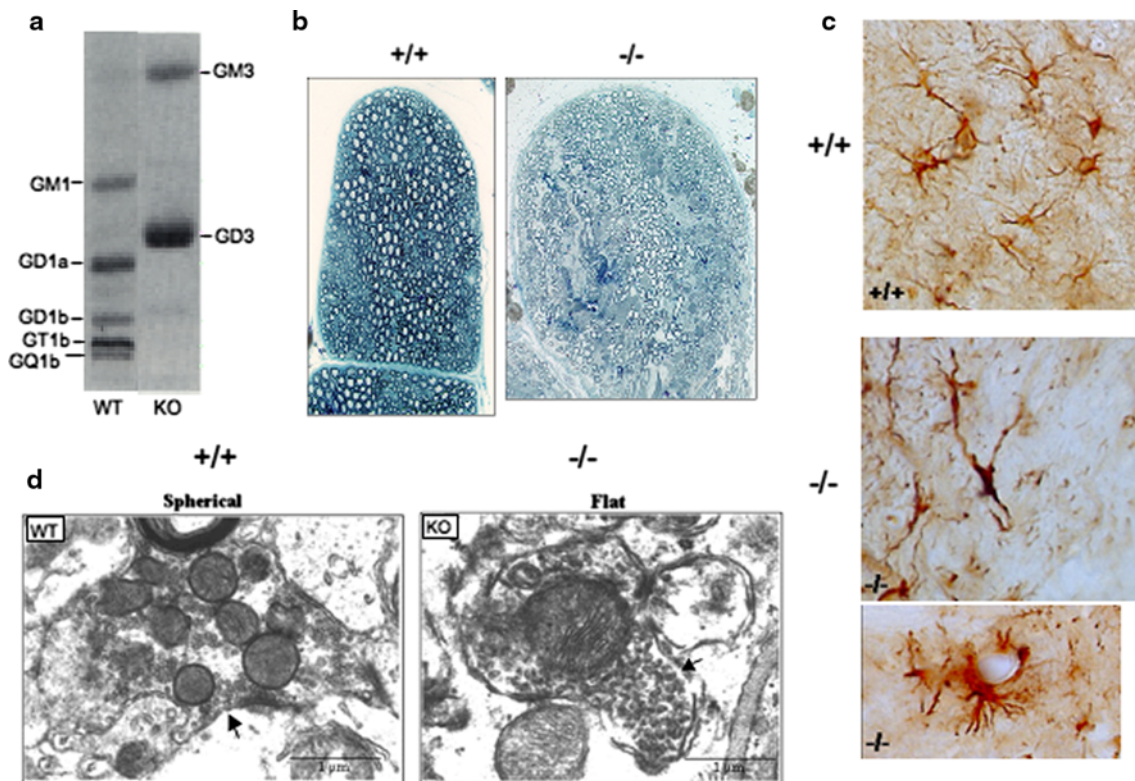
**Fig. 2** Neurodegenerations found in GM2/GD2 synthase knockout mice. **a** Ganglioside profiles as analyzed by TLC and resorcinol. **b** Sciatic nerves of 50 weeks old mice were shown. **c** Astrocytes observed in the spinal cord of wild type and GM2/GD2 synthase knockout mice. With immunohistostaining using an anti-GFAP antibody revealed abnormal astrocytes with thick and long protrusions. Some of them encircled blood vessels. *Top side* is normal astrocytes. **d** Morphological changes in synaptic vesicles of central terminal neurons are shown. In wild type, spherical and uniform types were dominant, while flat type vesicles were frequently found in KO tissues with other polymorphic and target-like shapes were also mixed. *Arrows* indicate representative examples

long period. Thus, it was shown that complex gangliosides are essential for the maintenance and repair of nervous tissues, while they are not indispensable for the neurodevelopment and differentiation [9].

Figure 2 showed changes of ganglioside profiles and marked neurodegeneration in sciatic nerves of GM2/GD2 synthase gene KO mice, where degenerative changes of sciatic nerves both in myelinated and non-myelinated fibers, and destroyed neuronal cells were detected. We also found deletion of cerebellum neurons, and morphological changes in synaptic vesicles and spine structures, suggesting the primary neurodegeneration and subsequent repair processes [9, 10]. Furthermore, proliferation and hypertrophic changes of glia were observed, suggesting the protective reaction against neurodegeneration caused by ganglioside deficiency.

These abnormal phenotypes detected in GM2/GD2 synthase KO mice certainly indicated ganglioside functions in the maintenance of the nervous tissue. However, it was true that observed phenotypic changes were much milder than expected [11]. This is because remaining glycosphingolipids in the nervous tissues of the individual KO

mice might play roles in the compensation for the functions of lost structures. This fact seems very troublesome in this kind of experiments. In GM2/GD2 synthase KO mice, amounts of the remaining GM3 and GD3 markedly increased, resulting in the maintenance of total ganglioside at almost equivalent levels with those in wild type mice. 9-O-acetyl GD3, that can be scarcely detected in normal tissues, was also accumulated in the mutant mouse brains [12].

It was also demonstrated that GD3 synthase KO mice could generate their offsprings with normal appearance, and they grew up normally despite of complete lack of all b-series gangliosides, A definite abnormal finding was an apparent decrease in the repair of damaged hypoglossal nerves [13]. Furthermore, even in GM3 synthase KO mice lacking all ganglio-series gangliosides, apparently normal mice were born and grew up. In these KO mice, asialo-series gangliosides markedly accumulated. Complete analyses on changes in the ganglioside composition in GM3 synthase KO mice were reported [14]. As a straightforward interpretation, we might think that the presence of ganglioside-back bone structures with similar

structures to the normally-found gangliosides are essential for the survival and functions of neurons regardless of the numbers and/or binding sites of sialic acids.

On the other hand, the fact that GlcCer synthase KO mice exhibited embyonal lethality due to the lack of all glycosphingolipids (except those of GalCer-derived structures) might indicate that the existence of molecules consisting of sugar chains (single sugar is not enough) bound to ceramide should be essential for cell survival and differentiation [15].

All these results suggested that differences only in the carbohydrate moiety do not induce severe defective phenotypes, and there is redundancy of functions among sugar chain structures. This fact, however, does not necessarily exclude the possibility that there are specific functions in the individual carbohydrate structures in glycolipids. In other words, we need to dissect molecular functions of glycosphingolipids based on their compositions by differentially evaluating their structures with hierarchy. First, carbohydrate functions which can be shared among glycolipids containing ceramide plus some sugar chains; 2nd, those which are defined mainly by the number of attached sialic acids; 3rd, those which are determined by the core structures and/or binding sites of sialic acids; 4th, those specifically exerted by the individual carbohydrate structures in glycolipids.

As for ganglioside functions in neuroregeneration, we have analyzed nerve repair activity by a hypoglossal nerve resection experimental model. To date, we analyzed regenerative activity in GD3 synthase KO, and GM2/GD2 synthase KO mice, and have demonstrated reduced regeneration activities depending on the degree of defective gangliosides [13, 16]. In particular, GM2/GD2 synthase KO mice showed marked reduction in the regenerative activity. As mechanisms for the reduction of regenerative activity, significantly decreased expression

levels of genes coding for neurotrophic factors and their receptors such as GDNF in the nucleus of hypoglossal nerves were demonstrated [16].

## Ganglioside Functions Elucidated by Double KO (DKO) of GM2/GD2 Synthase and GD3 Synthase

As described above, knockout of a single glycosyltransferase gene (except GlcCer synthase) could not verify clear significances of carbohydrate functions. Therefore, it was required to analyze phenotypes of KO mice in which gangliosides are thoroughly eliminated. In order to overcome this issue, we established double (D) KO mice, in which both GM2/GD2 synthase and GD3 synthase were disrupted, and have analyzed their phenotypes and mechanisms for the resulting neurological disorders. These DKO mice lacked all gangliosides except GM3. Therefore, they were named "GM3-only mouse" [17]. (Ganglioside composition in cerebellum of DKO was shown in Fig. 3a).

As expected, DKO of GM2/GD2 synthase and GD3 synthase genes showed severe neurodegeneration even in the early stage of life after birth. They also showed skin lesions with high frequency at 12 weeks after birth as shown in Fig. 3b. Elevated thresholds to the pain sensation in skin seemed to be a main cause for this refractory skin lesion [17]. In the behavior analyses, DKO showed exacerbating emotional disorders, sensory dysfunctions, reduced motor functions, and marked decrease in memory and learning functions with aging, suggesting that gangliosides are essential in the maintenance of nervous tissues [18]. In Fig. 3c, deletion of Purkinje cells in cerebellum of a 50 weeks old mouse was shown. In addition to these results, responses to injected oxotremorine revealed that DKO mice underwent lowered functions of muscarinic type acetylcholine receptors [19], suggesting
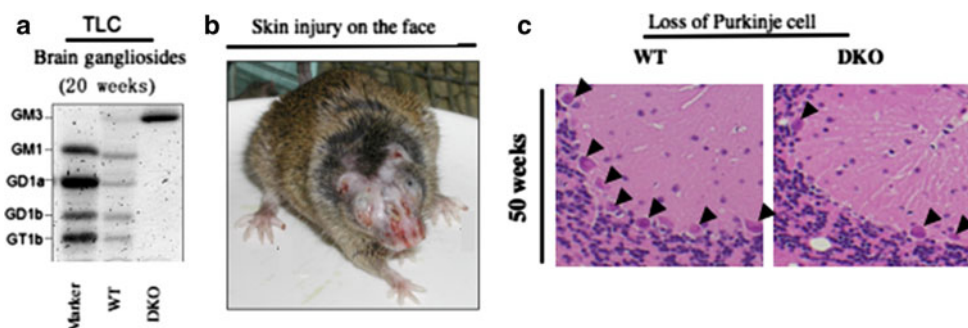


**Fig. 3** Skin lesions and neurodegeneration in double knockout mice. Phenotypes of double knockout mice (GM3-only mice) generated by mating GM2/GD2 synthase knockout and GD3 synthase knockout mice. **a** TLC patterns of gangliosides extracted from brain tissues of wild type and double knockout mice. While WT sample showed several ganglioside bands, double knockout samples showed only GM3 band. **b** Refractory skin lesion that emerged 12 weeks after birth are shown. Lesions at finger chips were also detected. **c** Degeneration of cerebella; general atrophy and deletion of Purkinje cells became marked with aging. Cerebellum tissues of 50 weeks old mice are compared

that expression levels of these receptor genes might be generally up-regulated for the compensation. Indeed, this was confirmed by quantitative (q-)RT–PCR. Therefore, continuously reduced functions of the muscarinic acetylcholine receptors might be main bases responsible for the progressive neurological dysfunctions in DKO mice [19]. Another group also reported DKO mice of GM2/GD2 synthase and GD3 synthase genes [20]. Main abnormal phenotype they found in the DKO mice was audiogenic seizure, suggesting over-sensitivity to sound stress. The differences in the phenotypes in the same DKO mice seemed to be due to differences in genetic backgrounds of ES lines used.

What are the mechanisms for the abnormal phenotypes, i.e. neurodegeneration, due to the defects of almost all gangliosides? This question seems easy to answer, but not necessarily easy to obtain a real answer. Here, we performed gene expression profiling using DNA microarray to compare the gene expression patterns in cerebella between DKO and wild type mice [21]. Among 25 genes that showed more than fivefold increase in the expression levels in DKO, there were more than one-third of genes belonging to inflammation-immune reaction-related genes. These results suggested that the inflammation might play roles in the nervous system of DKO mice. Increased expression of the inflammation-associated genes was also detected in GM2/GD2 synthase KO mice, although it was not marked.

On the other hand, complex KO of GM2/GD2 synthase and GM3 synthase, in which even GM3 was depleted, exhibited early death at a few weeks after birth probably due to marked neurodegeneration [22]. These results indicated that remaining GM3 in GM3-only mice had a critical significance in the maintenance of life. In this case, it is not clear whether inflammatory reactions were observed.

In the explanation of the abnormal phenotypes found in glycolipid-deficient mutant mice, we can not necessarily rule out the possibility that detrimental effects of the accumulated glycolipids cause neural dysfunctions in the individual KO mouse tissues. For example, GM3 and GD3 in GM2/GD2 synthase gene KO mice [8] may cause toxic effects on the brain functions, although it seems more likely that they compensate for roles of defective complex gangliosides. However, the comparison of double knockout mice of GM2/GD2 synthase/GD3 synthase [16] and those of GM2/GD2 synthase/GM3 synthase [22] clearly indicate that the presence of GM3 apparently enhances cell survival and activity of the nervous system.

## Neurodegeneration Is Induced Via Inflammation

Among the up-regulated genes in DKO mice that are associated with inflammation and/or immune reactions,

complement and its-related genes were included, suggesting the presence of the complement system activation. To confirm this point, time course of the expression levels of the complement-related genes and complement receptor genes were examined by q-RT–PCR as shown in Fig. 4a. Almost all genes showed up-regulation in DKO, and changes in the protein levels were also demonstrated. However, protein levels of those genes sometimes showed no marked changes or even decreased despite of increased levels of mRNA with aging. Taken together, over-consumption of the complement system might have occurred due to the marked complement activation. This was also supported by the increased levels of inflammatory cytokines such as IL-1α, IL-1β, and TNFα (Fig. 4b), and by marked proliferation of astrocytes and microglia. Consequently, in DKO brain tissues, inflammatory reaction due to the complement activation could be detected after 15 weeks old, and it was more enhanced with aging. Finally, these inflammations seemed to have induced neurodegeneration.

There have been a number of reports to indicate that inflammation is involved in the neurodegenerative diseases [23]. In particular, inflammatory reactions are thought to be one of causal or enhancing factors in Alzheimer disease [24, 25]. Evidences for involvement of the complement system in the neurodegeneration has been also reported in various diseases, and therapeutic trials with inhibition of the complement system have been also performed. It has been recently elucidated that besides systemic complement systems mainly regulated by liver, independent complement systems are also present and independently exerting their activities in nervous tissues [26]. The complement system is sometimes involved in the clearance of cell debris derived from developmental processes of neural tissues, leading to the maintenance of their intact functions [27]. On the other hand, a number of studies on the detrimental functions of the complement activation in the triggering of destruction of nervous tissues and subsequent neurodegeneration have been reported. In order to clarify whether complement activation is essentially involved in the neurodegeneration found in DKO, we established triple KO (TKO) by mating DKO with complement C3-deficient mice. Analyses of TKO mice revealed that majority of various inflammatory reactions, i.e. deposits of complements, increased inflammatory cytokines, and decreased weights of brains in DKO were restored close to the levels of wild type mice (Fig. 5). Namely, it was verified that inflammation and neurodegeneration found in DKO should be largely due to the complement activation. As for astrocyte proliferation, it was suggested that the astrocyte proliferation was induced independently from the complement activation, and by some unknown mechanisms.
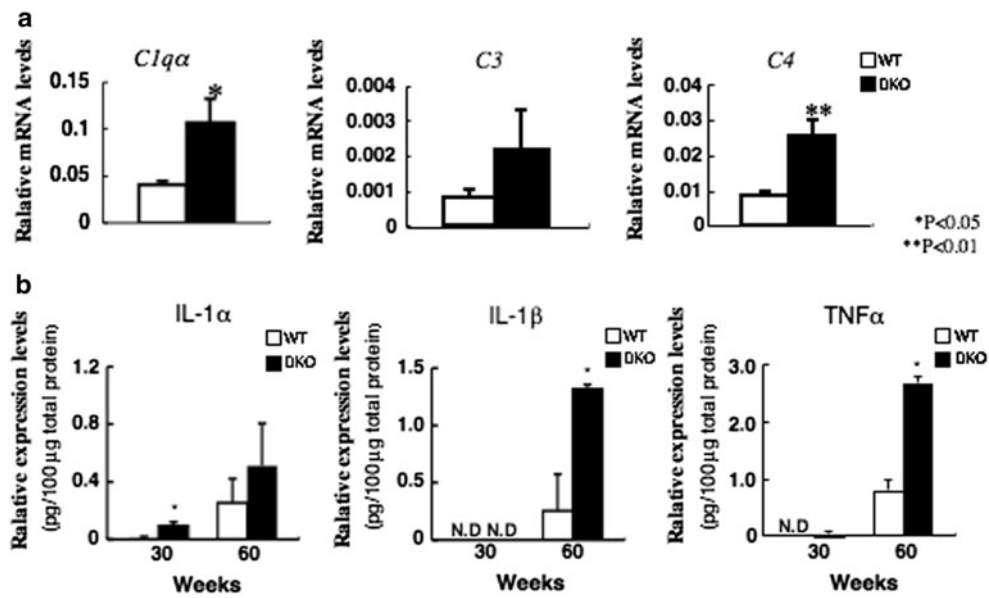
**Fig. 4** Complement activation and increased secretion of inflammatory cytokines found in double knockout mice. Since increased expression levels of complement system mRNAs were observed in cerebellum of double knock out in micro-DNA array, fifteen complement and it's relating genes were examined for their expression levels in wild type and double knockout mice. Some of the representative results including *C1qα*, *C3*, and *C4* are shown (**a**). These are results of 28 weeks old mice. Furthermore, ELISA of inflammatory cytokines were performed and the results are shown in **b**. All IL-1α, IL-1β, and TNFα showed marked increases particularly at 60 weeks after birth in double knockout mice
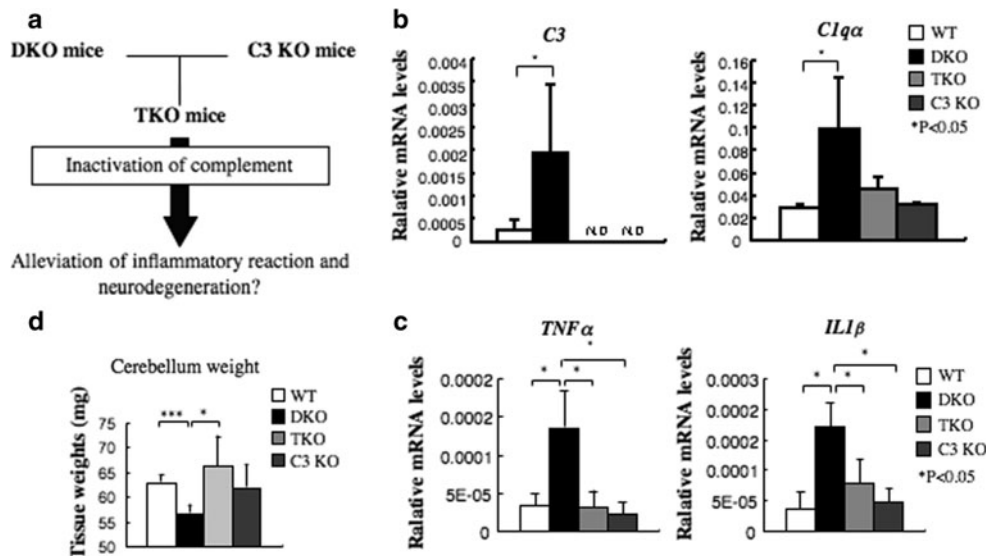


**Fig. 5** Alleviation of inflammation and neurodegeneration in triple KO mice: Complement activation is a main factor causing neurodegeneration. **a** To clarify the involvement of complement systems in the inflammatory reactions and neurodegenerations in double knockout mice, double knockout and C3-deficient mutant mice were mated, generating triple knockout mice. **b** In the triple knockout, not only *C3* but *C1qα* were decreased in the expression levels of mRNA. **c** Reduced inflammatory cytokines. The inflammation detected in double knockout mice was markedly alleviated. Therefore, it was clearly elucidated that the complement activation found in double knockout mice should be a main factor causing the inflammation, and subsequent neurodegeneration

Although I described above that accumulated glycolipids in the individual KO mice should not cause detrimental changes in the nervous system, accumulation of some glycolipids at extraordinary levels as shown in various storage diseases might bring about significantly toxic effects on cellular functions. For example, mutant mice disrupted of β-galactosidase or β-hexosaminidase showing tremendous accumulation of GM1 or GM2 ganglioside,

respectively, frequently exhibit inflammation in the nervous system [28, 29]. Similar findings were also observed in a patient's case [30]. NPC mice with accumulation of GM2 and GM3 as well as cholesterol also show inflammatory reactions in nerve tissues [31]. Thus, it seems very interesting to examine whether the complement activation is also involved in the inflammatory reactions in these storage diseases. Generation of complex KO mice including the complement system as shown in our experiments [21] would be of very much interest.

## Gangliosides Regulate Architectures and Functions of Lipid Rafts

What is the mechanism by which ganglioside deficiency induces complement activation? In order to investigate this issue, we analyzed the features of lipid rafts in the nervous tissues. Lipid rafts are microdomains in cell membranes, enriched in cholesterol, sphingolipids and GPI-anchored proteins. They form a solid and compact micro-structure consisting of lipids with less unsaturated fatty acids [32, 33]. Recently, lipid rafts have been considered to be important as sites where various biological processes such as endocytosis, cholesterol metabolism, infection of microorganisms, and various signal transductions occur. However, its concept and substantial basis have been unclear. We examined the distribution patterns of molecules that are believed to exist in lipid rafts by fractionating the Triton X-100 extracts from cerebella of DKO mice with sucrose density gradient ultracentrifugation. Consequently, it was strongly suggested that ganglioside deficiency triggers disorders in the architectures and functions of lipid rafts. GPI-anchored proteins as well as the marker for lipid rafts, such as flotillin-1 and caveolin-1, were found dominantly in non-lipid rafts in DKO [21], suggesting that dispersion of these molecules from lipid rafts occurred due to the changes in ganglioside composition.

These findings were confirmed by immunohistochemistry of cerebellum. Although CD55 is usually stained in membrane in normal tissues, it was also stained in cytoplasm with a diffuse pattern in addition to membrane regions in DKO. These results strongly suggested that fundamental structures of lipid rafts were basically destroyed.

Subsequently, we analyzed architectures of lipid rafts in various glycosylation- mutant mice using cerebellum tissues. The results revealed that various degrees of disorders in the raft architectures gave rise to depending on the intensities of ganglioside deficiencies [34]. As a conclusion, the disruption of glycolipid compositions might affect the formation and maintenance of lipid rafts, and might cause serious disorders in their regulatory functions of signaling in lipid rafts. In other words, the integrity of lipid rafts should be maintained based on the preferable compositions of glycosphingolipids, and thereby, normal cellular functions and tissue homeostasis can be maintained.

## Complement Activation Is Induced by the Disrupted Functions of Lipid Rafts

What is the mechanism by which ganglioside deficiency induces complement activation? In order to understand the mechanism, we need to consider the significance of complement regulatory proteins that are involved in the protection of host organs and tissues from the complement attack. The presence of complement regulatory factors such as CD55 and CD59 (GPI-anchored proteins) are essential in our bodies in order not to be injured by the complement system, i.e. one of main systems of the innate immunity. Thus, it is strongly suggested that disruption of the lipid rafts due to the ganglioside deficiency as described above should have disturbed correct localization and intact functions of the lipid rafts-resident molecules, such as complement regulatory proteins. Consequently, it has been demonstrated that the correct composition of gangliosides play important roles in the complement regulation by maintaining the integrity of lipid rafts based on the analyses of DKO mice. These results were summarized in Fig. 6.

On the other hand, the fact that proliferation of astrocytes observed in DKO was also observed in the single KO of GM2/GD2 synthase suggested that gangliosides have inhibitory functions in cell growth via the regulation of lipid rafts. For example, aspects of inhibitory functions of
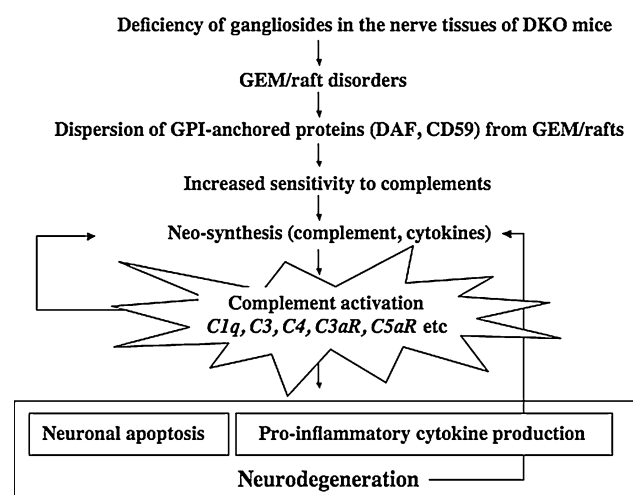


**Fig. 6** A Schema to indicate the mechanisms of neurodegeneration in DKO mice. Gangliosides deficiency induced inflammatory reactions of nerve tissues due to complement activation, leading to the neurodegeneration. DAF, CD55

gangliosides have been reported, i.e. inhibitory functions of GM3 [35, 36] against insulin receptors or EGF receptors, or suppressive effects of GM1 on PDGF receptors [37], and the secretion and activation of MMP-9 [38], which we reported. We need to consider the possibility that some other growth signals and/or excitatory signals than complement activation might be induced in the ganglioside-deficient mice.

## Conclusion

Abnormal phenotypes and analyses of their mechanisms revealed the significance of glycosphingolipids and suggested the possibility that they are involved in the regulation of various membrane molecules in a wide variety of body sites. In the brain tissues, gangliosides might be involved in the regulation of receptors such as neurotrophic factor receptors, adhesion receptors, neurotransmission factor receptors, muscarinic acetylcholine receptors, serotonin receptors, glutaminic acid receptors, and complement regulatory proteins, and play a integrative role of the nervous system. Therefore, it seems to become possible to artificially modulate the formation and functions of lipid rafts based on the modification of glycosphingolipid compositions.

As for the heterogeneity of lipid rafts, many investigators have recently predicted its presence [39]. But no definite evidences have been shown to date. Using electron microscopy, definite differences in the intracellular localization between GM1 and GM3 has been reported by our collegue [40]. Eventually, we hope to clarify the specific biological significances in the individual carbohydrate structures on glycolipids. Therefore, to us, it should be crucial to elucidate substantial bases for the heterogeneity of lipid rafts.

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