Gangliosides play pivotal roles in the regulation of complement systems and in the maintenance of integrity in nerve tissues

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Gangliosides are considered to be essential in the maintenance and repair of nervous tissues; however, the mechanisms for neurodegeneration caused by ganglioside defects are unknown. We examined gene expression profiles in double knockout (DKO) mice of GM2/GD2 synthase and GD3 synthase genes and showed that the majority of complement genes and their receptors were up-regulated in cerebellum in DKO mice. Inflammatory reactions were demonstrated in those tissues by measuring up-regulated inflammatory cytokines, indicating the presence of complement activation and inflammation as reported in Alzheimer’s disease. Immunoblotting of fractionated membrane extracts by sucrose density gradient revealed that complement-regulatory molecules such as decay-accelerating factor and CD59 were dispersed from glycolipid-enriched microdomain/rafts in DKO cerebellum. Immunohistostaining of these molecules showed disordered membrane localization. These results suggested that dysfunction of complement-regulatory molecules may be due to abnormal glycolipid-enriched microdomain/rafts that triggered complement activation, subsequent inflammation, and neurodegeneration in DKO mice. Generation of the triple KO mice lacking complement activity in addition to the two glycosyltransferases suggested that complement activation is involved in the inflammatory reactions and neurodegeneration caused by the ganglioside deficiency.

cerebellum | degeneration | inflammation | knockout | lipid raft

Gangliosides, sialic acid-containing glycosphingolipids, have been historically considered to be involved in the development, differentiation, and function of nervous systems in vertebrates (1). It has been recently clarified, however, based on studies of glycosylation-mutant animals, that gangliosides may mainly play roles in the maintenance and repair of nervous tissues (2, 3). Mice deficient in glycosyltransferases responsible for synthesis of gangliosides have exhibited degenerative changes mainly in the peripheral and central nervous systems (4, 5). GM2/GD2 synthase knockout (KO) mice showed mild neurological dysfunction at birth and progressive neurodegenerative changes (4–6). In contrast, combined KO of GM2/GD2 synthase and GD3 synthase (7) (double KO, hereafter abbreviated DKO) exhibited severe neurodegeneration with earlier onset and wider pathology distribution (8, 9). KO mice of glucosylceramidase synthase, in which Glc-Cer synthase was conditionally deleted in the brain after birth, also exhibited neurodegenerative changes (10). All of these observations suggested that a lack of gangliosides causes defects in maintenance of integrity of nervous tissues. While deficiency of some groups of glycolipids was shown to be associated with neurodegenerative changes, the mechanism of degeneration caused by the absence of certain glycolipids has not been demonstrated.

In various neurodegenerative diseases, particularly in Alzheimer’s disease (AD), one important factor considered to be involved in the death of neurons is local inflammation (11, 12). All components of the classical complement pathway have been identified in neurons (13), and this pathway has been reported to be activated in AD by fibrillar β amyloid peptide (14) or neurofibrillary tangles (15). The detection and activation of the alternative pathway in AD has also been reported (16). These results suggest that complement activation and inflammation is one of the mechanisms that may damage the brain in AD.

By analyzing gene expression in mice with KO of glycosyltransferases responsible for ganglioside synthesis we found that complement genes were up-regulated in nerve tissues. Further investigation revealed that both complement activation and inflammatory reactions are observed in the mutant mice, possibly based on disruption of membrane microdomains, that is, lipid rafts (17) or glycolipid-enriched microdomains (hereafter GEM/rafts). GEM/rafts have recently gained the interest of many biological researchers (18) for their putative roles in various biological processes (19).

We report here that neurodegeneration is associated with activated complement systems and subsequent inflammatory reactions in ganglioside-lacking mutant mice, which may be based on disrupted GEM/rafts. Involvement of the complement system in the inflammatory reaction and neurodegeneration was supported by mating DKO mice and C3-deficient mutant mice.

Results

Neurodegeneration Associated with Simultaneous Deletion of GM2/GD2 Synthase and GD3 Synthase. Double KO mice were generated lacking GM2/GD2 synthase and GD3 synthase genes (Fig. 1A). The cerebellum, containing only GM3 in TLC (Fig. 1B), weighed less in DKO mice at both 30 weeks (WT: 61.0 ± 5.6 mg, vs. DKO: 46.5 ± 8.7 mg) and 50–65 weeks (WT: 60.4 ± 5.7 mg vs. DKO: 48.9 ± 8.6 mg) (Fig. 1C). DKO mice displayed staggering gait (Movie S1). There was a clear loss of Purkinje cells in 30- (WT: 4.4 ± 0.1 vs. DKO: 3.0 ± 0.2) and 50-week-old (WT: 4.4 ± 0.2 vs. DKO: 2.8 ± 0.1) DKO cerebellum, although Purkinje cells in the 8-week-old DKO mice somewhat increased (WT: 4.7 ± 0.2 vs. DKO: 5.8 ± 0.1) (Fig. 1D and E). Moreover, progressive tremor and abnormal gait were observed in the DKO mice, suggesting that neurodegeneration occurred in the cerebellum.

Genes of Complement Components Were Up-Regulated in the Cerebellum of DKO Mice. To investigate the mechanisms for neurodegeneration in the DKO mice, a DNA microarray analysis were performed with mRNAs from cerebellum of 28- and 48-week-old mice. Among genes differentially expressed between WT and DKO mice, complement genes (C1qa, C1qb, C3, C4, and CD59) were...
found to be up-regulated in the nervous tissues (Table S1). C4 expression was markedly up-regulated in the cerebellum of 48-week-old DKO mice (WT: 1.37 vs. DKO: 5.00).

To precisely determine gene expression profiles, real time RT-PCR was performed with mRNAs from individual mice. Consequently, expression levels of C4 and C3αR were up-regulated in DKO mice compared to WT at all ages examined, except in 48-week-old females (Fig. 2A and Fig. S1).

The complement system functions in innate immunity and inflammatory reactions with sequential involvement of diverse components. Real time RT-PCR was performed for all complement system genes using mRNAs from the cerebellum. Expression levels of C1qa, C1qβ, C1qγ, C4, and C3αR were significantly up-regulated in DKO mice (Fig. 2B), suggesting that a majority of complement genes were up-regulated.

**Complement Gene Expression Was Up-Regulated Increasing with Time in the DKO Mice.** Because the pathology of DKO mice was exacerbated with aging, we examined chronological changes in the expression levels of C1qa, C3, C4, C3αR, and C5αR using mRNAs from mouse cerebellum and liver at various ages (Fig. 3). Expression levels of those genes clearly increased in DKO mice at 15 weeks of age, and the WT became definite with aging (Fig. 3 Left). Expression levels of complement genes were generally higher in the liver than in nerve tissues (Fig. 3 Right). These results suggested that up-regulation of complement genes took place during neurodegeneration in DKO mice. Actual protein levels and deposits of C4 in cerebellum were demonstrated (Fig. S2).

**Secretion of Inflammatory Cytokines in the Cerebellum of DKO Mice.** To investigate whether inflammation occurred in the cerebellum, mRNA expression levels of inflammatory cytokines (IL-1α, IL-1β, and TNFα) were measured in the nerve tissues. Expression of IL-1α was up-regulated in DKO mice at all ages (Fig. 4A). Expression of IL-1β and TNFα was also up-regulated in DKO mice, showing a tendency to increase with age. mRNAs of IL-6 and IFNγ were not detected in either type of mice.

In ELISA for the inflammatory cytokines, IL-1α showed an approximate 10-fold increase in 30-week-old and 2-fold increase in 60-week-old DKO mice (Fig. 4B). IL-1β increased approximately 6-fold in 60-week-old DKO mice, and TNFα increased approximately 3.4-fold in 60-week-old DKO mice. These results suggested that the cerebellum of DKO mice had an inflammatory reaction, exacerbated with age.

**DAF and GEM/Raft Markers Dispersed from GEM/Rafts in the Cerebellum of DKO Mice.** Complement-regulatory proteins such as decay-accelerating factor (DAF, or CD55) or CD59 are GPI-anchored proteins present in GEM/rafts, along with gangliosides. Therefore, it was predicted that GPI-anchored proteins undergo disorders due to altered GEM/rafts in the DKO mice.

The total protein levels of DAF were not different in the cerebellum of WT and DKO mice (shown in Fig. S3). Therefore, we examined alterations of GPI-anchored proteins as well as GEM/raft markers in the floating patterns inside/outside of GEM/rafts in the DKO mice. Triton X-100 extracts from cell membranes of the cerebellum were separated by sucrose density gradient centrifugation. We found that DAF, N-CAM, flotillin-1, and caveolin-1 largely dispersed from the fractions reported to contain GEM/rafts in the DKO mice (Fig. 5A).

Furthermore, we performed immunohistostaining to examine...
levels of five complement genes with real time RT-PCR and presented after livers of 4-, 15-, and 28-week-old mice were analyzed for the expression of DKO mice. mRNAs from the cerebella of 4-, 15-, 28-, and 48-week-old mice and granular cells of DKO mice was also present in the cytoplasm (Fig. 5B). The ratios of fluorescence intensities between membrane and cytoplasm (Fig. 5C) and the sharpness of the peaks (Fig. 5D) significantly decreased in the DKO mice (Fig. 5B Right). These results suggested that DAF existed in both plasma membranes and cytoplasm in DKO mice. Flotillin-1 and CD59 also stained distinctly when comparing DKO and the WT mice (Fig. 5E and Fig. S4, respectively).

Alleviation of the Inflammatory Reaction and Neurodegeneration in the DKO Mice by Genetic Disruption of Complement C3. To investigate whether the inflammatory reaction and neurodegeneration in DKO mice associated with complement systems, we generated triple KO (TKO) mice by mating DKO with C3 KO mice. In this study, 15- and 30-week-old mice were used, where the inflammatory reaction and neurodegeneration were already obvious.

As expected, expression levels of C3 were null in the TKO mice (Fig. 6A). Although expression levels of C1qa were significantly higher in DKO mice, they became almost equivalent in TKO and WT mice. Deposits of C1q and C3b/iC3b/C3c (active forms of C3) were examined by immunohistostaining. TKO mice showed no deposits of any component (Fig. 6B and Fig. S5), indicating that complement activation was completely suppressed in TKO mice.

By measuring mRNA expression levels of inflammatory cytokines (IL-1α, IL-1β, and TNFα), expression of IL-1β and TNFα was down-regulated in TKO mice (Fig. 6C), while IL-1α tended to decrease. Inflammatory reactions observed in DKO mice were absent in TKO mice. The weights of cerebellum, which were significantly lower in the DKO mice than in the WT mice, were normal in the TKO mice (WT: 63.0 ± 1.4 mg vs. DKO: 56.8 ± 1.7 mg vs. TKO: 66.3 ± 6.0 mg vs. C3 KO: 62.0 ± 4.6 mg) (Fig. 6D), suggesting the lack of neurodegeneration. The number of Purkinje cells in the cerebellum restored in the TKO mice, although there was a clear loss of Purkinje cells in DKO mice compared with WT mice at 30-week-old (WT: 4.4 ± 0.1 vs. DKO: 3.0 ± 0.2 vs. TKO: 3.8 ± 0.2 vs. C3 KO: 4.2 ± 0.1) (Fig. 6E).

These results suggested that the deficiency of gangliosides were associated with disorganized GEM/rafts and dysfunctions of complement-regulatory proteins in DKO mice, leading to abnormal activation of the complement system and enhanced inflammatory reaction, which may be linked to neurodegeneration.

Discussion

Besides KO mice of GM2/GD2 synthase, a number of mutant mice of glycosyltransferase genes responsible for the synthesis of glycolipids or their precursors have been reported in ref. 2. These

![Fig. 3. Complement genes were up-regulated with aging in the cerebellum of DKO mice. mRNAs from the cerebella of 4-, 15-, 28-, and 48-week-old mice and from livers of 4-, 15-, and 28-week-old mice were analyzed for the expression levels of five complement genes with real time RT-PCR and presented after correction by the GAPDH gene. Left column, expression levels of C1q, C3, C4, C3aR, and C5aR in the cerebellum. Right column, expression levels in the liver. Open diamond, WT; closed square, DKO. The number of mice examined was: WT n = 3, DKO n = 3; data are presented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

![Fig. 4. Inflammatory cytokines increased in the cerebellum of DKO mice with aging. (A) mRNAs from the cerebellum of individual mice were analyzed for the expression levels of cytokine genes with real time RT-PCR, and corrected by the mouse GAPDH gene. Open diamond, WT; closed square, DKO. The number of mice examined was: 4-, 15-, 30-, and 60-week-old WT n = 3, DKO n = 3. (B) The protein levels of IL-1α, IL-1β, and TNFα in the cerebellum of 30- and 60-week-old mice as analyzed by ELISA. The number of mice examined was: 30- and 60-week-old WT n = 3, DKO n = 3. **P < 0.01; ***P < 0.001. N.D., not detectable.

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KO mice were found to have almost normal brains and histological architectures when they were born, except KO mice of GcCer synthase (20). The majority of them exhibited degenerative changes in peripheral and central nervous systems after birth, indicating that gangliosides play roles in the maintenance of structures and functions of nervous tissues. However, definite mechanisms for neurodegeneration due to the loss of glycolipids have not been reported.

To investigate mechanisms for neurodegeneration caused by the lack of gangliosides, gene expression profiles in DKO nerve tissues were analyzed. Among genes showing up-regulation >3-fold in DKO significant numbers of inflammation- and immune-function-related genes such as S100A8 and Lrg1 as well as C4 and C3aR were identified. mRNAs of the majority of complement components showed up-regulation in the cerebellum of the DKO mice, while those in liver showed different behaviors (Fig. 3). These results suggested that the complement system in nerve tissues undergoes regulation distinct from that of systemic organs. Protein levels of complement components, however, did not clearly increase as the mRNAs did. Complement proteins in nerve tissues rather decreased with aging, suggesting that there is extensive consumption of complement components based on complement activation (21), and this effect appeared to become more serious with aging.

Basically, the complement system protects host health by attacking exogenous pathogens with or without association of antibodies (21). In addition to the systemic complement system, there are also complete features of the complement system in CNS; that is, complement factors, complement receptors (22, 23), and complement regulators (24). Not only glial cells such as astrocytes and microglia, but also neurons can generate these molecules (13, 22, 23).

In the last few decades, it has been demonstrated that the complement system is involved in diverse human neurodegenerative disorders such as AD (22). Complement activation has been considered to be positively involved in the progress of these diseases. In amyloid plaques and neurofibrill tangles, C1q and other components are deposited (14, 15), leading to complement activation. mRNAs of complement components are largely increased at the diseased regions (16, 25). Furthermore, an inhibitor of C1q attenuates AD pathology (26), suggesting that C1q exerts a detrimental effect on neuronal integrity, and activation of complement systems plays a central role in inflammation and subsequent neurodegeneration (11, 12).

In the DKO mice, almost all complement components underwent up-regulation, and the presence of obvious inflammatory reaction in the brain was demonstrated. The behavior of the complement system appeared similar to that found in AD. Thus, activation of the complement system and subsequent inflammation should be a critical factor exacerbating neurodegeneration in the DKO mice. However, the mechanisms for activation of the complement system leading to inflammatory reaction in the DKO mice were not clear, while it is well-known that accumulated Aβ causes astrocyte activation, cytokine production, and complement activation in AD (14, 15). Namely, the intermediate process between ganglioside defects and activation of the complement system and inflammation might be a key issue in the mechanism for neurodegeneration in the DKO mice.

One of the most important factors affecting complement activation and resulting neuronal damages are complement-regulatory proteins. In particular, CD59 has been well examined concerning its expression and functions in neuronal tissues. In fact, it is well known that expression levels of CD59 are largely suppressed in the diseased areas of AD (24). DAF is also a critical molecule for maintaining the integrity of tissues (27). Many of these complement-regulatory proteins are GPI-anchored proteins, and are enriched in GEM/raft fractions (28). Drastic changes in ganglioside compositions in the DKO mice might have induced profound modulation in the architectures and functions of GEM/rafts. Actually, even raft marker molecules such as flotillin-1 and caveolin-1 were found to be dispersed outside of GEM/rafts in the cerebellum of the DKO mice (Fig. 5).

Disorder of GEM/rafts in DKO brain tissues could bring about a disturbance in GPI-anchored protein functions. Consequently, the complement systems might not be well controlled, triggering proinflammatory reactions. Increased levels of proinflammatory cytokines in DKO nervous tissues further proved that inflammatory processes progressively occurred due to complement activation based on the disrupted regulatory system against self-attack. Why complement activation was induced mainly in nerve tissues is a most intriguing issue in need of clarification. First, the original ganglioside levels expressed should be exceptionally high in nervous tissues compared to other tissues, resulting in more serious damage by withdrawal mechanisms. Second, nervous tissues might be more susceptible to a lack of complement-regulatory molecules as shown in human fetal neurons (29). Furthermore, disturbed distribution of GEM/raft-residing molecules was demonstrated mainly by biochemical ways. Coexistence of these molecules on the living cells or tissues should be proved by direct methods in the future as suggested in ref. 30.

As a double-edged sword, the complement system has been considered to play both positive and negative roles (31) in terms of disease control. Besides roles in the induction of inflammation with complement activation and eventual neurodegeneration
GD2 synthase, GD3 synthase and C3 genes were successfully
sive neurodegeneration. To clarify this issue, TKO mice of GM2/
subsequent inflammatory reactions due to reduced functions of
the complement system in chronic disorders (36).

Critical. Many complement inhibitors have been applied to treat
served (22, 36). The causes of complement activation might be
or spatiotemporal differences of the pathological processes ob-
tissues have been explained based on differences in disease phases
neurodegeneration.

Aberrant innervation might be important in protection from
functions in the clearance of cell debris, and refinement of
prominent neurodegeneration. Thus, the complement system
these cases, inhibition of the complement system results in
unnecessary degraded cell components or the refinement of
physiological roles of complements such as the elimination of

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Fig. 6. Alleviation of the complement activation, inflammation, and neurodegeneration in the DKO mice by disruption of complement C3 gene. (A) Expression levels of complement mRNAs from the cerebella of 15-week-old mice were analyzed for C1qα and C3 genes with real time RT-PCR. Relative expression levels are presented after correction by the GAPDH gene. The numbers of mice examined were: 15-week-old WT n = 5, DKO n = 4, TKO n = 3, C3 KO n = 3. (B) Deposits of C1q in the cerebella of 15-week-old WT, DKO, TKO, and C3 KO mice were analyzed with anti-C1q mAb in combination with Alexa Fluor 488-conjugated anti-rat IgG. Fluorescence intensity (mean pixel × total area) of complement staining was measured by digital image analysis. Number of section area examined was: 15-week-old WT n = 6, DKO n = 6 TKO n = 6, C3 KO n = 6; data are presented as mean ± SD. The thickness of sections was 7 μm. (Scale bar, 20 μm.) (C) Expression levels of cytokine mRNAs (IL-1α, IL-1β, and TNFα) were analyzed with real time RT-PCR using RNAs from the cerebellum of individual mice. Relative expression levels are presented after correction by the mouse GAPDH gene. The number of mice examined was: 15-week-old WT n = 4, DKO n = 4, TKO n = 3, C3 KO n = 3. [Scale bar, 50 μm (A and B).] Thickness of sections, 7 μm. (D) Wet weights of the cerebella of 15-week-old WT, DKO and TKO mice as measured in Fig. 1. Numbers of tissues examined were: WT n = 6, DKO n = 5, TKO n = 3, C3 KO n = 3; data are presented as mean ± SD. (E) Cerebellar sections of 30-week-old mice stained with HE. Arrowheads indicate Purkinje cells. The numbers of Purkinje cells were counted and presented as in Fig. 1. The numbers of mice examined were: 30-week-old WT n = 2, DKO n = 2 TKO n = 2, C3 KO n = 2. Data are presented as mean ± SD. (Scale bar, 50 μm.) * P < 0.05; ** P < 0.001.

functions in development (32, 33), neurogenesis in adults (34), and protection in disease-susceptible mutant mice like Tg/AB have been reported in ref. 35. In particular, physiological roles of complements such as the elimination of unnecessary degraded cell components or the refinement of the inflammatory reaction have been reported in ref. 32. In these cases, inhibition of the complement system results in prominent neurodegeneration. Thus, the complement system functions in the clearance of cell debris, and refinement of aberrant innervation might be important in protection from neurodegeneration.

These contradictory functions of complement systems in nervous tissues have been explained based on differences in disease phases or spatiotemporal differences of the pathological processes observed (22, 36). The causes of complement activation might be critical. Many complement inhibitors have been applied to treat acute inflammation or brain injury, suggesting beneficial roles of the complement system in chronic disorders (36).

In the case of the DKO mice, complement activation and subsequent inflammatory reactions due to reduced functions of regulatory proteins may be a causal factor bringing about progressive neurodegeneration. To clarify this issue, TKO mice of GM2/GD2 synthase, GD3 synthase and C3 genes were successfully generated. Resulting phenotypes of the TKO mice demonstrated that complement activation may be involved in the complement deposits, increased secretion of inflammatory cytokines and reduced cerebellum weights (degeneration).

Although there have been a number of reports on the dynamic, quantitative, and qualitative changes in GEM/rafts during stimulation of cells, cell–cell interaction, infection of microorganisms, and endocytosis (37), disordered and altered distribution of GEM/rafts has never been reported to date. As for caveolae, the lack of its main component, caveolin-1, results in the disappearance of the characteristic concave morphology (38) and functional defects (39). In the case of GEM/rafts, there are no definite morphological features, and ganglioside GM1 has been used as a specific raft marker (19). In our recent studies, GM1 seemed to be a functional molecule to regulate signals via PDGF or NGF (40). In the DKO mice analyzed here, all gangliosides other than GM3 were lost. Therefore, the effects on the membrane environment should be very serious. In addition to the effects of altered GEM/rafts on GPl-anchored proteins, other functional proteins such as neurotrophic factor receptors (3) and integrins (41) might be also affected by the altered GEM/rafts.

In the DKO mice, changes in the floatation patterns of DAF and flotillin-1 suggested that microdomains as a stable platform for anchoring raft-residing molecules were disrupted. Ambiguous staining patterns of these molecules in tissue sections also
suggested that the membrane structure underwent fundamental transfiguration in the DKO mice. However, correlation between intensity of the transfiguration of GEM/rafts and degree of ganglioside defects needs to be clarified. Fine structural disorders in the membrane, including GEM/rafts, in the DKO tissues also remain to be investigated.

Experimental Procedures

Mice. The mutant mice used in this study were generated and maintained in our laboratory. GM2/GD2 synthase KO (6) and GD3 synthase KO (7) mice were mated; that is, heterozygotes of individual mutants were mated, and genotypes of the offspring were screened for the two genes as described in ref. 6. All experimental protocols were approved by the animal experimental committee of the Graduate School of Medicine in Nagoya University along the guidelines of Japanese government, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1966).

Antibodies. Antibodies used for Western immunoblotting and those used for immunohistochemistry were described in the SI Text. Chemiluminescence detection and immunofluorescence detection reagents were also in SI Text.

Primers. Primers used for real time RT-PCR were designed according to Primer 3 Input as shown in Table S2.

Real Time RT-PCR. WT and DKO mice were perfused with PBS. The cerebella and livers were isolated from mice and homogenized in TRIzol (Invitrogen). Total RNA was extracted following the manufacturer’s protocol. RT-PCR was performed as described in ref. 3.

Western Immunoblotting. The cerebellum and spinal cord were isolated after perfusion with PBS from mice and were homogenized in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 50 mM NaF, 1 mM NaVO4, 1% Triton X-100, 200 mM PMSE, and 0.01–0.02 TruLum aprotinin). The lysates were pelleted by centrifugation at 8,000 × g for 60 min at 4 °C. The supernatant was centrifuged at 18,000 × g for 90 min at 4 °C, and clarified lysates were used for immunoblotting. Immunohisto staining. WT and DKO mice were perfused with PBS and then 4% paraformaldehyde in 0.1 M phosphate buffer. The cerebellum and spinal cord were removed, postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C, then replaced with 10% sucrose for 6 h, 15% sucrose for 6 h, 20% sucrose for 6 h, and embedded in O.C.T. compound (Sakura Finetechical) and frozen in liquid nitrogen. Frozen sections were cut at longitudinal orientation for the cerebellum and at dorso-ventral orientation for the spinal cord, and were cut into 7- to 10-μm-thick sections on a cryostat (LEICA, CM3050S) at –20 °C. For immunohistochemistry, tissue slices were stained by antibodies and were sealed with PermaFlour aqueous mounting medium (Thermo). Coverslips were mounted and observed by light microscopy or confocal fluorescence microscopy (FLUOVIEW FV500; Olympus).

ELISA. ELISA measurement of levels of cytokines in tissues and fractionation of extracts with sucrose gradient ultracentrifugation are described in SI Text.

Statistical Analysis. Values obtained in the experiments were analyzed for significance with Student’s t test. When P values were <0.05, they were considered significant.

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Supporting Information

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SI Text

Mice. Complement component 3-defective mice (B6.129S-C3m1Crr/J) were obtained from The Jackson Laboratory. GM2/GD2 synthase and GD3 synthase genes KO mice were mated with C3 KO mice, and genotypes of the offspring were screened for the three genes. Genotypes in C3 KO mice were screened as described (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocolId=407).

Extraction of Glycolipids and TLC. Glycolipid extraction and TLC were performed as described in ref. 6.

DNA Microarray. The cerebella and spinal cords isolated from 28and 48-week-old mice (n = 3) were homogenized in TRizol, and total RNA was extracted from tissues following the manufacturer’s protocol (Invitrogen). Comparison of the gene expression profiles was performed using pooled RNAs from three mice. DNA gene chip analysis was performed depending on custom analysis services (KURABO).

Hematoxylin-Eosin (H&E) Staining. WT and DKO mice were perfused with PBS (PBS) and then 10% formalin neutral buffer solution (Wako), and were postfixed with 10% formalin neutral buffer solution. The cerebellum isolated from mouse was cut in ~3-mm sections and embedded in paraffin using Tissue Tek VIP5 junior (Sakura) after dehydration and paraffin penetration. Blocked cerebellum was cut into 3-μm sections and used for H&E staining. H&E-stained slices of the cerebellum were observed by light microscopy.

Antibodies. Antibodies used for western immunoblotting were as follows: anti-mouse C1q mAb (rat IgG1) (Hycult Biotechnology), anti-β-actin mAb (Sigma), goat anti-mouse DAF (Santa Cruz Biotechnology), anti-rat flotillin-1 mAb (mouse IgG1) (BD Biosciences), anti-mouse Thy-1.2 mAb (rat IgG1) (AbD Serotec), anti-mouse NCAM mAb (rat IgG2a) (Abcam), and rabbit anti-human Caveolin-1 (Santa Cruz Biotechnology). Chemiluminescence detection was performed using HRP-conjugated rabbit anti-rat IgG (H+L) (ZYMED Laboratories), sheep anti-mouse IgG (Amersham Biosciences), horse anti-goat IgG (H+L) (Vector Laboratories). Antibodies used for immunohistochemistry were as follows: anti-mouse C1q mAb (rat IgG1) (Hycult Biotechnology), anti-mouse C3b/C3b/C3c mAb (rat IgG1) (Hycult Biotechnology), anti-mouse DAF mAb (mouse IgG) (a kind present from Dr. Mizuno at Nagoya University), anti-human CD59 mAb (mouse IgG2b) (Bio Vendors), rabbit anti-human flotillin-1 (Santa Cruz). Immunofluorescence detection reagents were purchased from Invitrogen; that is, Alexa Fluor 488-goat anti-mouse IgG2b, Alexa Fluor 488-goat anti-rat IgG, Alexa Fluor 555-goat anti-mouse IgG1, Alexa Fluor 488-goat anti-rabbit IgG, or Alexa Fluor 546-goat anti-mouse IgG.

Fractionation of Extracts with Sucrose Density Gradient Ultracentrifugation. All steps were performed on ice. After perfusion with PBS, tissues were isolated and minced in PBS containing 1 mM PMSF followed by nitrogen cavitation. Nitrogen-cavitated samples were centrifuged at 1,000 rpm for 10 min at 4 °C. The supernatant fractions were centrifuged at 100,000 × g for 1 h at 4 °C (Beckman Coulter, Optima MAX-E), and the obtained pelleted membrane fractions were resuspended in 1.2 mL MNE buffer (25 mM Mes, pH 6.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1 mM PMSF, and 0.01–0.02 TIU/mL aprotinin) containing 1% Triton X-100, and treated with 10 strokes of a Dounce homogenizer (Iuchi). Resuspended samples (1 mL) were then added to an equal volume of 80% sucrose in MNE buffer in an ultracentrifuge tube and overlaid with 3 mL discontinuous sucrose gradient, consisting of 30% sucrose in MNE buffer (2 mL) and 5% sucrose in MNE buffer (1 mL). Ultracentrifugation was performed at 100,000 × g for 16 h at 4 °C in an Optima MAX-E (Beckman Coulter). Fractions containing 0.5 mL each were obtained from the top to the bottom of the tube.

ELISA. After perfusion with PBS, tissues were isolated, homogenized, and sonicated in lysis buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 50 mM NaF, 1 mM NaVO4, 1% Triton X-100, 1 mM PMSF, and 0.01–0.02 TIU/mL aprotinin). The lysates were centrifuged at 8,000 × g for 60 min at 4 °C, then the supernatants were centrifuged at 18,000 × g for 90 min at 4 °C. Clarified lysates were used for ELISA. Levels of IL-1α, IL-1β, and TNFα proteins were determined using assay kits for mouse IL-1α/IL-1F1, mouse IL-1β/IL-1F2 ELISA, or mouse TNFα/TNFβIA (R&D Systems). The assay was performed according to the manufacturer’s instructions.

Genes of Complement Components Were Up-Regulated in the Cerebellum of DKO Mice. To precisely determine gene expression profiles, real time RT-PCR was performed with mRNAs from individual mice. Consequently, expression levels of C3αR were up-regulated in DKO mice compared to WT at all ages examined, except in 48-week-old females (Fig. S1).

Complement Proteins Increased in Young DKO Mice. We performed immunoblotting to investigate the protein level of C1q which is essential in the complement system and up-regulated in the DKO mice as described above. C1q protein levels in the cerebellum of 15-week-old DKO mice were increased 3-fold compared with WT (Fig. S2A), although they were almost equivalent at 30 weeks (Fig. S2B), and decreased at 60 weeks in DKO mice (Fig. S2C) even though C1q mRNA levels were up-regulated with aging. To determine whether C1q was activated in DKO mice, deposits of C1q were analyzed with immunohistostaining. At both 15 (Fig. S2 D and F) and 30 weeks (Fig. S2 E and G) deposits of C1q increased in the cerebellum of DKO mice, suggesting progressive activation of C1q at 15 weeks and thereafter.

CD59 Was Dispersed from GEM/Rafts in the Cerebellum of DKO Mice. CD59 also stained distinctly when comparing DKO and the WT mouse (Fig. S3).

Alleviation of the Inflammatory Reaction in the DKO Mice by Genetic Disruption of Complement C3. To investigate whether the inflammatory reaction in DKO mice associated with complement systems, we generated triple KO (TKO) mice by mating DKO with C3 KO mice. Deposits of C3b/C3b/C3c (active forms of C3) as well as C4 were examined by immunohistostaining. TKO mice showed no deposits (Fig. S4), indicating that complement activation was completely suppressed in TKO mice.
mRNA levels of C3aR were up-regulated in the cerebellum of DKO mice. Expression of the C3aR mRNA levels were analyzed by real time RT-PCR and presented after correction with GAPDH gene. The number of mice examined was: 28-week-old male WT n = 3, DKO n = 3; 28-week-old female WT n = 3, DKO n = 6; 48-week-old male WT n = 3, DKO n = 3; 48-week-old female WT n = 3, DKO n = 5; data are presented as mean ± SD. **, P < 0.01; ###, P < 0.001.
Fig. S2. Complement C1q levels in the cerebellum as analyzed by immunoblotting and immunohistostaining. (A–C) Western immunoblotting of C1q in the cerebellum was performed using total lysates from 15-, 30-, and 60-week-old WT and DKO mice. The intensity of the bands was corrected with that of β-actin and presented as mean ± SD. The numbers of mice examined was: 15-week-old WT n = 2, DKO n = 2; 30-week-old WT n = 3, DKO n = 3; 60-week-old WT n = 2, DKO n = 3. Each mouse is designated as W or D plus number. (D–G) Deposits of C1q in the cerebella of 15- (D and F) and 30-week-old (E and G) WT and DKO mice were analyzed with anti-C1q mAb and Alexa Flour 488-conjugated anti-rat IgG. Data were presented as number of detected spots per 800 × 800-μm space of the individual layers of cerebellum section (F and G). The thickness of sections was 7 μm. (Scale bar, 20 μm (D and E).) The number of mice examined was: 30-week-old WT n = 3, DKO n = 3; data are presented as mean ± SD. * P < 0.05; ** P < 0.01; *** P < 0.001.
Fig. S3. Total protein levels of DAF were not different in the cerebellum of WT and DKO mice. Western immunoblotting of DAF and flotillin-1 was performed using whole lysates of the cerebellum to examine total protein levels. DAF bands were corrected by those of β-actin. The number of mice examined was: 30-week-old WT n = 3, DKO n = 3; data are presented as mean ± SD. No significant differences were found.
The CD59 distribution pattern was altered in the cerebellum of DKO mice. Confocal microscopic imaging of granular cells of 15- and 60-week-old cerebellum labeled with an anti-CD59 mAb, demonstrating that CD59 was stained on both cell membranes and cytoplasm of the DKO mice, while WT mice showed staining only on cell membranes. (Scale bar, 10 μm.)
Fig. S5. Alleviation of the complement activation and inflammation in the DKO mice by disruption of complement C3 gene. Deposits of C3b/iC3b/C3c in the cerebella of 15-week-old WT, DKO, TKO, and C3 KO mice were analyzed with anti-C3b/iC3b/C3c mAb in combination with Alexa Flour 488-conjugated anti-rat IgG. Fluorescence intensity (mean pixel × total area) of complement staining was measured by digital image analysis. Number of section area examined was: 15-week-old WT n = 6, DKO n = 6, TKO n = 6, C3 KO n = 6; data are presented as mean ± SD. The thickness of sections was 7 μm. (Scale bar, 20 μm.) Data are presented as mean ± SD. (Scale bar, 50 μm.) * P < 0.05; *** P < 0.001.
Movie S1. Behavior of a DKO mouse (Upper) and a WT mouse (Lower).

Movie S1 (MP4)
Table S1. Expression levels of complement components using DNA microarray

<table>
<thead>
<tr>
<th>Tissue and weeks</th>
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<th>WT (expression)*</th>
<th>DKO (expression)*</th>
<th>Ratio</th>
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<td></td>
<td>C4</td>
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*Expression levels of genes in WT and DKO mice were quantified based on normalized signal intensities (see Experimental Procedures in the main text) obtained from DNA microarray.*
**Table S2. Sequences of primers used for real time RT-PCR**

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*Primer sequences for the primers used for real time RT-PCR were designed according to Primer 3 Input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.www.cgi).

*Primers used for TKO mice.*