Mice Expressing Only Monosialoganglioside GM3 Exhibit Lethal Audiogenic Seizures*

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Gangliosides are a family of glycosphingolipids that contain sialic acid. Although they are abundant on neuronal cell membranes, their precise functions and importance in the central nervous system (CNS) remain largely undefined. We have disrupted the gene encoding GD3 synthase (GD3S), a sialyltransferase expressed in the CNS that is responsible for the synthesis of b-series gangliosides. GD3S−/− mice, even with an absence of b-series gangliosides, appear to undergo normal development and have a normal life span. To further restrict the expression of gangliosides, the GD3S mutant mice were crossed with mice carrying a disrupted GalNAcT gene encoding β1,4-N-acetylgalactosaminyltransferase. These double mutant mice expressed GM3 as their major ganglioside. In contrast to the single mutant mice, the double mutants displayed a sudden death phenotype and were extremely susceptible to induction of lethal seizures by sound stimulus. These results demonstrate unequivocally that gangliosides play an essential role in the proper functioning of the CNS.

Gangliosides are glycosphingolipids that contain sialic acid (reviewed in Ref. 1). They are found on the external leaflet of the plasma membrane on eucaryotic cells and are most abundant in the central nervous system (CNS) where they represent the major sialoglycoconjugate. Because of their dramatic changes in expression during neuronal development and differentiation (3–6), as well as their prominence in the mature CNS, gangliosides have long been assumed to have fundamental roles in CNS development and function.

In the ganglioside biosynthetic pathway (1) (see Fig. 2A), lactosylceramide serves as the core structure. The first ganglioside synthesized, GM3,2 is produced by the transfer of an α,2,3-linked sialic acid residue to lactosylceramide. Subsequently, GM3 can be modified by the action of β1,4-N-acetylgalactosaminyltransferase (GalNAcT, EC 2.4.1.92) to produce GM2 and other complex gangliosides. Alternatively, GM3 can be modified by the action of GD3 synthase (CMP-sialic acid: GM3 α,2,8-sialyltransferase, EC 2.4.99.8) to produce the disialoganglioside GD3, which diverts the pathway to the synthesis of b- and c-series gangliosides. Gene targeting in mice has been a particularly fertile approach for uncovering the functions of gangliosides in the CNS. Disruption of the GalNAcT gene (7) blocks the synthesis of complex gangliosides and results in the expression of only the simple gangliosides GM3 and GD3. Surprisingly, these mutant mice are viable, with a normal life span and a CNS that is largely intact both morphologically and functionally (8, 9). These mice do, however, exhibit an age-related dysmyelination process that is associated with axonal degeneration (10). The mechanism for dysmyelination may be the absence of neuronal ganglioside ligands for myelin-associated glycoprotein (MAG) resulting in myelin instability. Ultimately, motor defects are observed in aged, 12-month-old GalNAcT−/− mice, suggesting a role for complex gangliosides in long-term CNS maintenance (11).

We have now produced mice with a disrupted GD3 synthase (GD3S) gene (12), of which expression is relatively high in the CNS. Even though these mice lack the disialylated b-series gangliosides in their CNS, they show no overt phenotype. By combining the GalNAcT and GD3S mutant alleles, we have established double knockout mice (DKO) that express only monosialylated GM3 as their major CNS ganglioside. In contrast to each of the single knockout mice, the DKO mice show a sudden death phenotype and a severe CNS disturbance that is manifested by an exquisite susceptibility to lethal, sound-induced seizures. These results provide compelling evidence for the critical and essential role of gangliosides in CNS function.

EXPERIMENTAL PROCEDURES

Targeted Disruption of the Mouse GD3S Gene and Generation of Mutant Mice—To generate the GD3S knockout mice, a genomic fragment was isolated from a 129Sv strain library (Stratagene, catalog no. 946306) containing the fifth exon of the 5-exon GD3S gene (13). For knocking-in the LacZ reporter gene and targeted inactivation of the GD3S gene, a LacZ-neo (neomycin-resistant gene) cassette (14) was inserted into the GD3S gene at the critical intron-exon boundary (15). The resulting allele was then sequenced to confirm that the genomic sequence was correct. The GD3S−/− mice were phenotypically indistinguishable from wild-type littermates. Electron microscopic examination of brain sections from GD3S−/− animals showed the absence of complex gangliosides, but the presence of simple gangliosides in all tissues examined. In contrast, brain sections from GD3S+/- mice showed a greater relative abundance of complex gangliosides in comparison to simple gangliosides. Both GD3S+/- and GD3S−/− mice were on the C57BL/6 background and were maintained as described (16).

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1 The abbreviations used are: CNS, central nervous system; DKO, double knockout mice; MAG, myelin-associated glycoprotein; GD3S, the gene encoding GD3 synthase; GalNAcT, the gene encoding β1,4-N-acetylgalactosaminyltransferase; TLC, thin layer chromatography; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; kb, kilobase pair; PBS, phosphate-buffered saline; E, embryonic day (e.g. E11.5).

2 The nomenclature for gangliosides follows the system of Swennerholm (2).
inserted into the BamHI site of the fifth exon. The resulting targeting vector contained an ~11-kb GD3S 5’ fragment and a 2.2-kb GD3S 3′ fragment separated by the LacZ-neo cassette (Fig. IA). The LacZ coding region is preceded by an internal ribosomal entry sequence. Therefore, targeted insertion generates a bicistronic transcription unit in which the expression of the β-galactosidase reporter protein is under the control of GD3S transcriptional regulatory elements. The herpes simplex virus thymidine kinase (TK) gene was positioned outside the homologous sequence to deter random integration events. Targeting of TC1 embryonic stem cells, we obtained clones with a homologously recombined GD3S allele. Targeted embryonic stem cells were electroporated into ES cells, and the recombined allele was readily detectable in the same extracts (Fig. 1A).

We next determined the embryonic expression pattern of the GD3S gene in mice, we created a targeting vector that would both render GD3 synthase inactive and allow monitoring of the GD3S locus expression (Fig. IA). The targeting vector was designed to introduce a LacZ reporter gene into the last exon of the GD3S gene. This targeting strategy would cause an early termination of the GD3 synthase protein, resulting in the elimination of the highly conserved sialyl S motif, a critical portion of the catalytic domain (22). Removal of the sialyl S motif from the GD3 synthase in this manner renders the enzyme inactive (16, 23). After electroporation of the targeting vector into embryonic stem cells, we obtained clones with a homologously recombined GD3S allele. Targeted embryonic stem cells were used to create chimeric mice and, subsequently, heterozygous mice. Crossbreeding of the heterozygous mice resulted in the birth of homozygous mutant mice (Fig. 1B) at the expected Mendelian frequency, indicating an absence of embryonic lethality associated with this mutation.

GD3 synthase activity was not detectable in extracts of GD3S−/− brain (Fig. 1C). However, GM3 synthase, the sialyltransferase that catalyzes the preceding step of ganglioside biosynthesis, was readily detectable in the same extracts (Fig. 1C).

RESULTS AND DISCUSSION

Generation of GD3 Synthase Knockout Mice—To disrupt the GD3S gene in mice, we created a targeting vector that would both render GD3 synthase inactive and allow monitoring of the GD3S locus expression (Fig. 1A). The targeting vector was designed to introduce a LacZ reporter gene into the last exon of the GD3S gene. This targeting strategy would cause an early termination of the GD3 synthase protein, resulting in the elimination of the highly conserved sialyl S motif, a critical portion of the catalytic domain (22). Removal of the sialyl S motif from the GD3 synthase in this manner renders the enzyme inactive (16, 23). After electroporation of the targeting vector into embryonic stem cells, we obtained clones with a homologously recombined GD3S allele. Targeted embryonic stem cells were used to create chimeric mice and, subsequently, heterozygous mice. Crossbreeding of the heterozygous mice resulted in the birth of homozygous mutant mice (Fig. 1B) at the expected Mendelian frequency, indicating an absence of embryonic lethality associated with this mutation.

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We next determined the embryonic expression pattern of the GD3S gene by whole mount staining of heterozygous and homozygous mutant embryos with X-gal. At E11.5, very strong LacZ expression was found in developing spinal cord. The eyes and the top of the metencephalon were also positive (not shown). Throughout later developmental stages, E13.5–E17.5, GD3S expression was readily detectable in the developing CNS (Fig. 1D).

As would be predicted from the location of GD3 synthase in the biosynthetic pathway of gangliosides (Fig. 2A), the brain ganglioside pattern of GD3S−/− mice was devoid of the b-series structures, GD1b and GT1b, and was dominated by the

Ganglioside Mutant Mice

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a-series gangliosides, GM1 and GD1a (Fig. 2B). The absence of b-series gangliosides further documents the disruption of the GD3S gene and validates the ganglioside synthesis scheme proposed by Sandhoff and colleagues (1, 24). The GD3S−/− mice were viable and fertile, had normal growth, and were without gross behavioral abnormalities. Histologic examination of brains from GD3S−/− mice did not reveal any obvious abnormalities. The GD3S−/− mice did not show observable demyelination as determined by Luxol Fast Blue staining of brain sections.

Establishment of Mice That Express Only GM3 GalNAcT— knockout mice express predominantly GM3 and GD3 gangliosides in their CNS due to the absence of β1,4-N-acetylgalactosaminyltransferase, yet they have fundamentally normal nervous system development and a normal life span (8, 9). We further narrowed the expression of ganglioside structures by crossing the two types of ganglioside mutant mice. Mice doubly mutant for the GalNAcT and GD3S genes were born at the expected Mendelian frequency. As predicted from the biosynthetic pathway of gangliosides (Fig. 2A), elimination of both transferases in the DKO mice resulted in the expression of the GD3S−/− mice as their major CNS ganglioside (Fig. 2B). Although roughly similar amounts of total brain ganglioside were present in wild-type and mutant mice, DKO mice contained markedly reduced levels of lipid-linked sialic acid in brain compared with wild-type and single mutant mice (Fig. 2C).

After their weaning we noted that the DKO mice had an extremely high mortality rate. We followed the life span of a group of DKO mice and found that by 13 weeks of age about 92% during the observation period (36 weeks). We searched for pathological changes in the DKO mice. We examined brain sections by Nissl, and Luxol Fast Blue staining but could not discern any gross pathological changes, including neuronal cell loss or significant demyelination. Histological examination was done with mice after timed sacrifice and after sudden death. It had been noted previously that expression of MAG, which binds certain gangliosides, was reduced in the GalNAcT mutant mice, presumably because of a deficiency of ganglioside ligands for MAG (10). We found that the single mutant GD3S mice had a level of MAG similar to wild-type mice (not shown). This finding can be explained by the presence of GD1a (Fig. 2, A and B), a relatively high affinity MAG ligand (25). In the DKO mice, MAG expression was similar to that of the single mutant GalNAcT−/− mice (not shown), presumably because of a comparable deficiency of MAG ligands in the two types of mutant mice.

Again, as had been noted in the GalNAcT−/− mice, the male DKO mice displayed a defect in sperm maturation resulting in sterility (not shown) (8, 26). No pathologic abnormalities were found in visceral organs in the DKO mice.

Double Mutant Mice Are Sensitive to Sound-induced Seizures— We noticed that some of the DKO mice showed apparently spontaneous and/or handling-induced seizures. In one case, a mouse died after such a handling-induced seizure attack. Therefore, we hypothesized that the reason for sudden death in the DKO mice may be enhanced susceptibility to lethal seizure. Sound is a classic means of inducing seizures in certain susceptible strains of mice (27). We used a relatively mild sound stimulus, key jangling, to test the DKO and single mutant mice for sensitivity to audiogenic seizure. We found that the double mutant mice were exquisitely sensitive to seizure induction by this method (Fig. 3B). The typical seizure began with a brief wild running phase, followed by a clonic-tonic seizure. In most cases the seizure resulted in death. All DKO mice tested were induced to have a clonic-tonic seizure. Ultimately, 9 of 10 DKO mice died during tonic phase, apparently because of respiratory arrest. None of the wild-type or GD3S−/− mice showed any seizure response to the sound stimulus. One of the nine GalNAcT−/− mice responded with wild running activity and a clonic seizure, but the episode did not progress further to lethality.

Why Are Mice That Express Only Monosialoganglioside GM3 Exquisitely Susceptible to Lethal Seizures?— Seizure susceptibility in mice has been shown to result from mutations in a large number of genes with diverse functions, including a variety of proteins involved in neuronal cell signaling (28). Gangliosides have been proposed to act as modulators of transmem-
GD3S may be a result of abnormal CNS development. Although it is also possible that the seizure susceptibility in DKO mice composition during the differentiation of neurons (3–6). Thus, neuronal cell signaling and seizure susceptibility.

Lipid-enriched membrane microdomains, resulting in impaired complement could alter the properties of these glycosphingolipids. Lyn, and with the glycosylphosphatidylinositol-anchored signaling protein, TAG-1, in glycosphingolipid-enriched microdomains. It is also of interest that mice deficient in Fyn kinase, another Src family kinase, are susceptible to audiogenic seizures (33). In the DKO mice, the undersialylated ganglioside complement could alter the properties of these glycosphingolipid-enriched membrane microdomains, resulting in impaired neuronal cell signaling and seizure susceptibility.

A role for gangliosides in the development of the CNS has been suggested based on the striking changes in ganglioside composition during the differentiation of neurons (3–6). Thus, it is also possible that the seizure susceptibility in DKO mice may be a result of abnormal CNS development. Although histologic examination of DKO mutant brains revealed no obvious abnormalities, subtle defects cannot be ruled out.

Finally, our findings may have relevance for human epilepsy, a disorder that burdens about 1% of the population (28). Our results raise the possibility that mutations altering the ganglioside synthesis pathways may be factors in contributing to some forms of this disorder.

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![Fig. 3. Life span and audiogenic seizures of ganglioside mutant mice. A. groups of mice (n = 9) of each of the genotypes were monitored for survival from an age of 4 weeks until 35 weeks. B. mice from each genotype (wild-type [WT], n = 5; GalNAcT−/−, n = 9; GD3S−/−, n = 5; GD3S/GalNAcT−/−, n = 10) were exposed individually to a sound stimulus created by jangling a set of keys for 5 s. The behavior of the mice was observed for the following: wild running, clonic seizure, tonic seizure, and death. *, one GalNAcT−/− mouse responded to the sound stimulus with wild running and a clonic seizure but not a tonic seizure or death. **, p < 0.001; χ² test.](image-url)