



# Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system

(gene knock-out/development/ $\beta$ 1, 4GalNAc transferase)

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**ABSTRACT** Gangliosides, sialic acid-containing glycosphingolipids, are abundant in the vertebrate (mammalian) nervous system. Their composition is spatially and developmentally regulated, and gangliosides have been widely believed to play essential roles in establishment of the nervous system, especially in neuritogenesis and synaptogenesis. However, this has never been tested directly. Here we report the generation of mice with a disrupted  $\beta$ 1,4-*N*-acetylgalactosaminyltransferase (GM2/GD2 synthase; EC 2.4.1.92) gene. The mice lacked all complex gangliosides. Nevertheless, they did not show any major histological defects in their nervous systems or in gross behavior. Just a slight reduction in the neural conduction velocity from the tibial nerve to the somatosensory cortex, but not to the lumbar spine, was detected. These findings suggest that complex gangliosides are required in neuronal functions but not in the morphogenesis and organogenesis of the brain. The higher levels of GM3 and GD3 expressed in the brains of these mutant mice may be able to compensate for the lack of complex gangliosides.

Gangliosides have been considered to play important roles in the development and differentiation of the nervous system (1–3), since they are abundantly expressed in the nervous system of many vertebrates (4, 5). Gangliosides are synthesized by consecutive addition of monosaccharides to ceramide by multiple glycosyltransferases in the Golgi apparatus (6–8) and are then transferred to the outer leaflet of the plasma membrane. Among the many glycosyltransferases,  $\beta$ 1,4 GalNAc-transferase ( $\beta$ 1,4 GalNAc-T; GM2/GD2 synthase; EC 2.4.1.92) plays an important role in biosynthesis of almost all complex gangliosides (Fig. 1) (9, 10, <sup>††</sup>). Expression of this gene was markedly increased at late stages of development (12–14), suggesting roles for complex gangliosides in the neuritogenesis and synaptogenesis.

To address the role of gangliosides in neuronal development and physiological events in the neural tissue, we generated mice with a disruption of the  $\beta$ 1,4 GalNAc-T gene by homologous recombination in mouse embryonic stem (ES) cells.

## MATERIALS AND METHODS

**Gene Targeting and Generation of  $\beta$ 1,4 GalNAc-T  $-/-$  Mice.** The mouse  $\beta$ 1,4 GalNAc-T was cloned from BALB/c mouse genomic library using a 2.1-kb *Xba*I fragment of mouse cDNA clone pTm3-5 (15) as a probe. The targeting plasmid was constructed containing a neomycin-resistant gene inserted

into the exon 4 as shown in Fig. 2A. The targeting vector (24 nM) was linearized with *Not*I and was mixed with ES cell suspension ( $1 \times 10^7$ ), then electroporated at 0.25 kV, 960  $\mu$ F, using a Bio-Rad GenePulser. Forty-eight hours after electroporation, G418 was added to the medium at the concentration of 150  $\mu$ g/ml. After 7–8 days, the G418-resistant clones were isolated and subjected to screening for homologous recombination by PCR. The sense primer was 5'-TCGTGCTTTACGGTATCGCCGCTCCCGATT-3' in 3' terminus of PGK neo, and the antisense primer was 5'-GGGTGTGGCGGCATACATCT-3' in the intron of the  $\beta$ 1,4 GalNAc-T gene. The reaction was started one cycle of 95°C (2 min), 55°C (1 min), 74°C (5 min), thereafter 35 cycles of 94°C (1 min), 60°C (30 sec), 74°C (1.5 min) were used. Homologous recombinant clones gave a 1.1-kb fragment. Two chimeric males derived from ES cell lines D-120 and G-193, respectively, transmitted the  $\beta$ 1,4 GalNAc-T mutation to progeny. Mice heterozygous for the disrupted  $\beta$ 1,4 GalNAc-T gene were mated, and homozygous mutant progeny were identified by PCR and Southern blot analysis of DNA isolated from mouse tails.

**TLC and Enzyme Assay of  $\beta$ 1,4 GalNAc-T.** Glycolipids were extracted as described (16). Briefly, lipids were extracted by chloroform/methanol at ratios of 2:1, 1:1, then 1:2 sequentially. Glycolipids were isolated by a Florisil column after acetylation, then neutral and acidic fractions were separated by DEAE-Sephadex (A-50) column chromatography. The enzyme activity of  $\beta$ 1,4 GalNAc-T was measured according to the method described (17). The membrane fractions were prepared as described by Thampoe *et al.* (18). The enzyme products were isolated by C18 Sep-Pak cartridge (Waters) and analyzed by TLC and fluorography as described (17). The enzyme activity of  $\beta$ 1,3 GalNAc-T [*Gb*Ose4Cer synthase; globotetraosyl-ceramide (globoside) synthase] was measured as described (19).

**Neuraminidase Treatment.** Neuraminidase digestion of gangliosides were performed as described (20).

**Morris Water-Maze and Other Behavior Tests.** The mice were subjected to the Morris water-maze test for spatial learning at the age of 10 weeks as described (21). The water-maze test was conducted twice a day for 3 days. One trial consisted of starting the mice at four points 90° apart and allowing them to swim and escape onto a hidden platform in the tub. The pillar holding the platform was fixed at the center of one quadrant of the circular tub (60 cm). The top of the

Abbreviations: GalNAc-T, GalNAc-transferase; ES cells, embryonic stem cells; SEP, somatosensory evoked potential; SSEP, spinal SEP; *Gb*Ose4Cer, globotetraosyl-ceramide (globoside); wt, wild type.

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<sup>††</sup>Ganglioside nomenclature is based on that of Svennerholm (11): GM2, GalNAc $\beta$ 1,4(NeuAc $\alpha$ 2, 3) Gal $\beta$ 1,4Glc-Cer; GM1, Gal $\beta$ 1,3GalNAc $\beta$ 1,4(NeuAc $\alpha$ 2, 3)Gal $\beta$ 1,4Glc-Cer.

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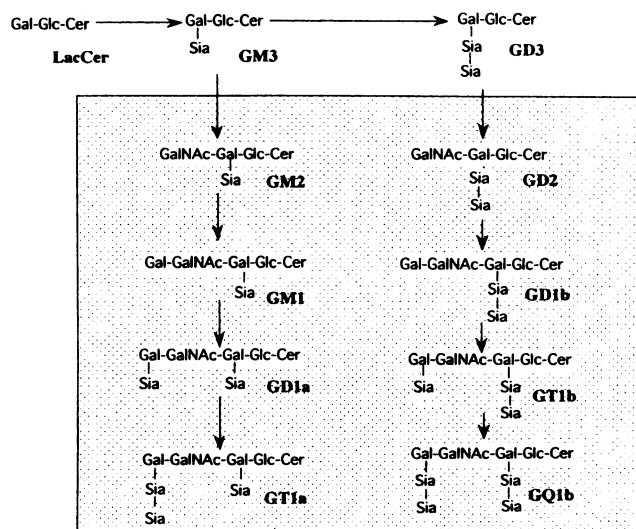


FIG. 1. Proposed pathway of ganglioside synthesis. The shaded box indicates gangliosides biosynthesis of which is blocked in  $\beta$ 1,4 GalNAc-T gene lacking mice. Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Sia, sialic acid; LacCer, lactosylceramide.

platform (5 cm in diameter, 29 cm high) sat 1 cm below the surface of the water, which was mixed with milk for camouflage. The time from the release of the mouse from each starting point until the mouse stood and/or escaped onto the platform was measured to calculate a mean for each trial. The measurement was terminated when the time exceeded 120 sec. The other tests for behavior, i.e., hindlimb reflex, spontaneous motor activity (22), flinch hearing (23), and inclined plane test (24), were performed as described. The statistical analysis was carried out with the Statistical Computer Package (SAS Institute, Cary, NC) using the analysis of covariance.

**Somatosensory Evoked Potential (SEP).** For determination of mouse S1 cortical SEP, mice were anesthetized with pentobarbiturate. They were placed in a stereotactic frame provided with a head holder consisting of a palate plate. For SEP, a 5- to 6-mm-diameter trepanation hole with a center 1.0 mm caudal and 2.0 mm lateral to the bregma was made to allow ample access to the parietal cortex (25). This procedure was established by 44 wild-type (wt) mice. Then, the indicated numbers of wt, heterozygous, and homozygous mice were studied with same method when the mice were 10 weeks old. Using the same mice, spinal SEP (SSEP) was recorded on the L2-3 interspace. For recording, the needle was placed extradurally and served as described (26). Initial peaks were readily identified by visual inspection of the recorded responses. To measure the peripheral myelinated conduction velocity, sciatic nerve volley was also recorded distal on the spinal cord. Stimulation for evoked potentials consisted of square wave pulses 0.2 msec in duration at a frequency of 2/sec. Stimulus intensity was adjusted to 10–20% above the voltage at which the maximum amplitude of the initial peak of the evoked response was observed. Each recorded response consisted of the average of 16 or 64 sweeps. The hindlimb was extended, and the distance between the stimulating cathode and each recording electrode was measured. In each evoked potential, we measured the latency of the initial peak of each response and calculated the conduction velocity. Statistical analysis and comparison was done using conduction velocity. Two-tailed, unpaired Student's *t* test was used to compare values obtained for each of the responses in different groups.

**RESULTS**

**Lack of Complex Gangliosides by Targeting Mutation of the  $\beta$ 1,4 GalNAc-T Gene.** The  $\beta$ 1,4 GalNAc-T gene was disrupted

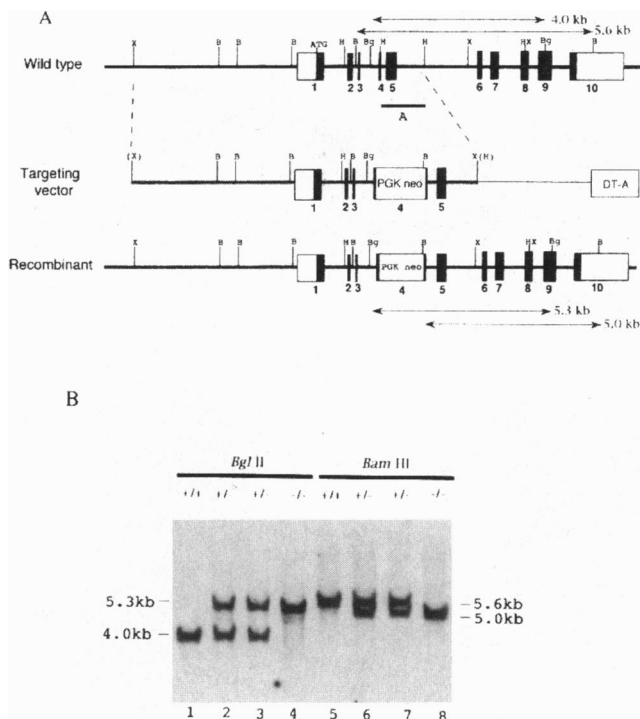


FIG. 2. (A) Strategy for disruption of the mouse  $\beta$ 1,4 GalNAc-T gene. "Wild type" represents the normal genomic structure of mouse  $\beta$ 1,4 GalNAc-T gene. Exons 1–10 are shown as a box on the line, with filled boxes representing coding regions. The translation initiation site is indicated as ATG. The middle line is the "Targeting vector" which was linearized at a unique *NotI* site. In this vector, neomycin-resistant gene with phosphoglycerate kinase-1 gene promoter and without poly(A)<sup>+</sup> addition signal (PGK neo) was inserted into the *HindIII* site in exon 4. The diphtheria toxin A fragment (DTA) gene with MC1 promoter was ligated on the 3' terminus across the vector backbone for negative selection. The lower line labeled "Recombinant" shows the structure of the  $\beta$ 1,4 GalNAc-T gene after a correct targeting event. PCR was used in the identification of homologous recombinants. The bold line labeled "A" represents the 800-bp *HindIII* fragment used as a probe to identify gene targeting events. Predicted sizes (kb) of *BglII* and *BamHI*-digested fragments hybridizing to this probe are shown. Abbreviations for restriction enzyme sites: X, *XhoI*; B, *BamHI*; H, *HindIII*; Bg, *BglII*. (B) Example of Southern blot analysis of F2 mice. The genomic DNA extracted from the tail was digested with *BglII* or *BamHI*, and hybridized with the shown probe under high stringency. Using *BglII* or *BamHI* digested DNA, the wild-type allele gave a 4.0-kb, 5.6-kb band and the recombinant allele gave a 5.3-kb and 5.0-kb band, respectively. Lanes 1 and 5 indicate wild-type, lanes 2, 3, 6, and 7 indicate heterozygous, lanes 4 and 8 indicate homozygous mutant mice-derived samples.

by inserting a neomycin-resistant gene in exon 4 as described (27). As shown in Fig. 2B, Southern blot analysis of F<sub>2</sub> mice indicated that knockout mice homozygous for the  $\beta$ 1,4 GalNAc-T gene had been successfully generated. As shown in Fig. 3A and B, TLC analysis of the acidic glycolipids demonstrated a lack of GM2 or complex gangliosides, such as GM1, GD1a, GD1b, and GT1b, in the liver or brain of the mutant mice. Instead, increased GM3 or GD3 and GM3 levels were found in the liver or brain of mutant mice, respectively. The identity of the putative GD3 band was confirmed by TLC immunostaining with an anti-GD3 monoclonal antibody (data not shown). Neuraminidase treatment of the brain samples (Fig. 3C) was also used to identify the gangliosides. A majority of the bands in the wt sample were converted to GM1, whereas no GM1 was detected in the homozygote-derived sample. Minor bands in homozygote brain gangliosides disappeared after neuraminidase treatment, suggesting that they were of the lacto- or neolacto- series. These results were in accordance

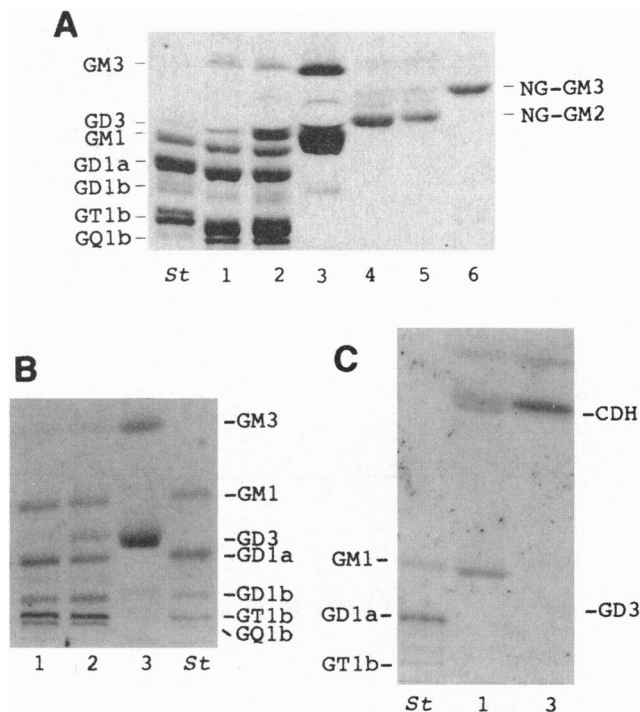


FIG. 3. (A) TLC of acidic glycosphingolipids from brain and liver of normal, heterozygous, and homozygous mutant mice (12 weeks old). In St, 5  $\mu$ g of bovine brain ganglioside mixture was applied as a standard. Lanes: 1 and 4, wt; 2 and 5, heterozygote; 3 and 6, homozygote. Lanes 1-3 are brain gangliosides and 4-6 are liver gangliosides. Resorcinol spray after TLC of the acidic fractions derived from 15 mg (wet) tissues. NG- means *N*-glycolylneuraminic acid. The solvent system used was chloroform/methanol/2.5 N  $\text{NH}_4\text{OH}$  (60:35:8). (B) TLC of brain gangliosides as in A except that one-third of the amount in A was applied and a solvent system of chloroform/methanol/0.22%  $\text{CaCl}_2$  (55:45:10) was used. (C) TLC of brain gangliosides from wt (lane 1) and homozygote (lane 3) mice after treatment with neuraminidase. Solvent system was as in B. At least three experiments were performed independently, and representative data are shown.

with the reported pathway of ganglioside synthesis by Sandhoff *et al.* (28). The  $\beta$ 1,4 GalNAc-T activity in homozygous mutant mice was not detectable in both liver and brain, confirming that the targeted gene disruption resulted in a null allele. Heterozygous mutants showed intermediate activity, i.e., 44.4% (brain) and 41.6% (liver) of that in normal littermates (Fig. 4A). The  $\beta$ 1,4 GalNAc-T activity in the mixture of extracts from wt and homozygous mice brain indicated that these mutants did not contain any inhibitors (Fig. 4B).  $\beta$ 1,3 GalNAc-T did not alter in the mutant mice as shown in Fig. 4C.

**Normal Histogenesis in Brain.** In histological analysis, brain tissues were examined with special attention since gangliosides are greatly enriched in vertebrate neural tissues. As shown in Fig. 5A, the brain of the mutant mice had almost normal size, weight, and shape, being indistinguishable from that of wt mice. The following regions or sites were carefully examined and were found to be morphologically normal by light microscopic examination: frontal, temporal, and occipital cortices; striatum; corpus callosum; hippocampus; third and lateral ventricle; plexus choroideus; cerebellum with nucleus dentatus; fourth ventricle; and brain stem. As shown in Fig. 5B-E, the cortex also showed normal density of pyramidal neurons and lamination. There was normal myelination of the white matter. The gross architecture and CA1-CA3 pyramidal and other neurons of the hippocampus and cerebral cortex were normal. The size and structure of the cerebellum were also normal (Fig. 5F and G). Gangliosides are enriched in synaptic membranes and have been implicated to play an important role in synaptic

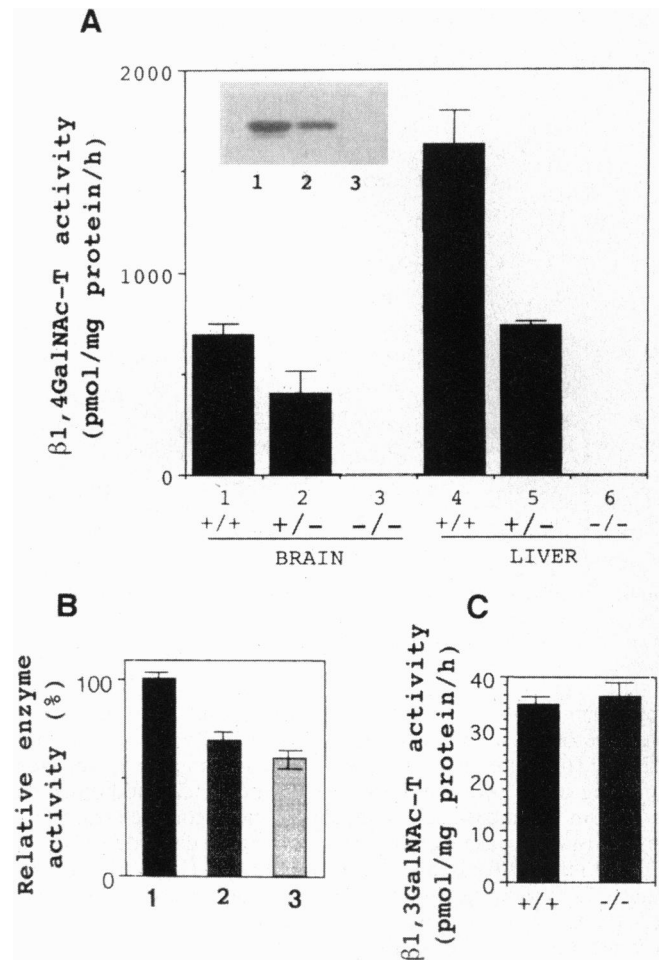
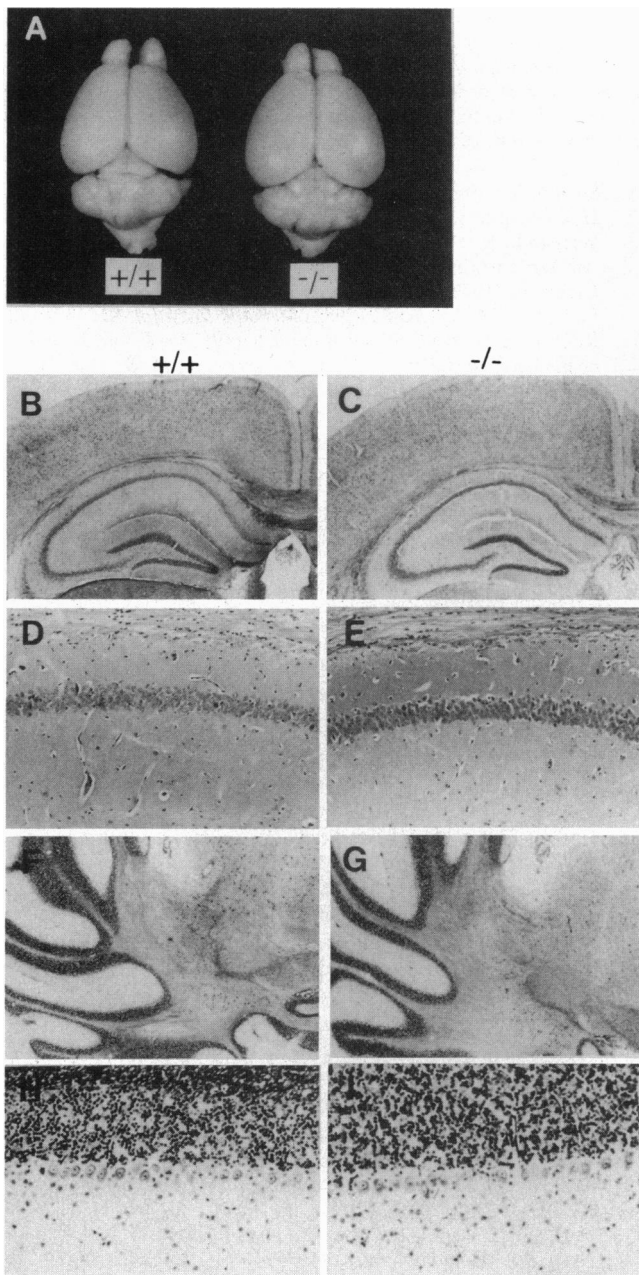


FIG. 4.  $\beta$ 1,4GalNAc-T enzyme activity in tissues from normal, heterozygous, and homozygous mutant mice. Membrane fractions were prepared from at least three mice for each group and the enzyme activities were measured as described in *Materials and Methods*. (A)  $\beta$ 1,4 GalNAc-T enzyme activity in brain and liver of mice as indicated. Inset shows products from enzyme reaction using brain extracts from wt (1), heterozygote (2), and homozygote (3) mice (12 weeks). (B) Relative  $\beta$ 1,4 GalNAc-T activity in 200  $\mu$ g (1) and 100  $\mu$ g (2) of the extract from wt brain and in the mixture of 100  $\mu$ g each of wt and homozygote extract (3). (C)  $\beta$ 1,3 GalNAc-T activity in the brain of +/+ and -/- type mice. The conditions for enzyme reaction were as described in *Materials and Methods*. (Bars = mean  $\pm$  SD.)

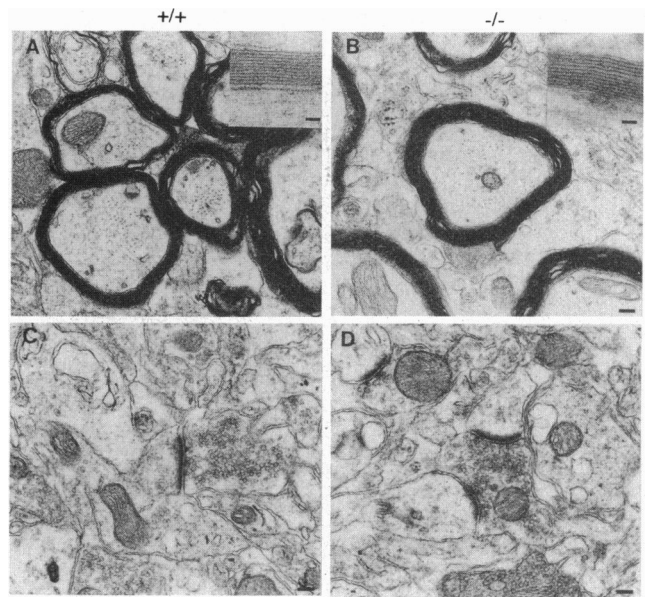
transmission (29, 30). Possible involvement of gangliosides in the process of myelination have been also suggested (31, 32). However, electron microscopical investigation of several brain tissues also showed normal structures in myelinated fibers and synapses as shown in Fig. 6.

**No Apparent Abnormality in Gross Behavior.** Homozygous mutant mice were apparently normal in gross behavior; they suckled and fed normally. No sign of ataxia was observed, and they displayed normal hindlimb reflex and postural changes. They were also normal in spontaneous motor activity and inclined plane test. No defect was also apparent in swimming ability and in flinch hearing. Furthermore, we tested for behavior abnormalities in the mutant mice with a special focus on the activity of memory and learning, since the  $\beta$ 1,4 GalNAc-T gene is mainly expressed in the hippocampus, dentate gyrus, cerebral cortex, cerebellar Purkinje cells, and mitral cells of olfactory bulb in the mouse brain tissue (12, 13). As shown in Fig. 7, the Morris water-maze test revealed no definite delay of learning in the mutant mice compared with wt mice.



**FIG. 5.** Intact morphology of brain tissues from complex gangliosides-lacking mice. (A) Photograph of brains from a wt (left) and the mutant (right) mouse (12 weeks). Microphotograph of brain sections from a wt (B, D, F, and H) and mutant (C, E, G, and I) mouse. B and C show a coronal section of hippocampus and cerebral cortex stained with Klüver-Barera's method (magnification,  $\times 20$ ). D and E show the hippocampal pyramidal cells (magnification  $\times 100$ ). F and G show a parasagittal section of the cerebellar cortex (magnification,  $\times 20$ ), and H and I are high magnification of F and G to show the detailed architecture of the cerebellum (magnification,  $\times 100$ ). More than five mice were examined and showed essentially same results.

**Some Defects in Neural Conduction Velocity.** We also examined the effects of ganglioside deficiency on myelination and synaptic transmission by electrophysiological approaches. Evoked potentials were recorded at the pial surface of the contralateral S1 somatosensory cortex and at the lumbar spine (L2) extradurally after stimulation of the peripheral tibial nerve at the Achilles tendon level. Representative evoked responses in wt and homozygote mice were shown in Fig. 8A and C. The conduction velocities calculated in individual groups were shown in Fig. 8B and D. No significant differences

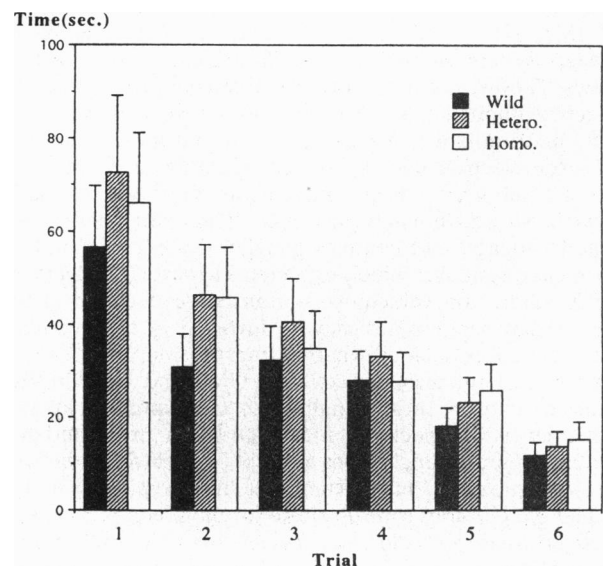


**FIG. 6.** Electron micrograph of myelins and synapses. Myelin formation in the brain of 12-week-old wt (A) and of the mutant mice (B). Bars = 200 nm. Insets show similar myelin layers by hypermagnification. Bars = 50 nm. Intact synapse formation in the mutant mice (D) comparing with that in wt mice (C) was shown. (Bars = 200 nm.)

were detected in L2 spinal record and the sciatic nerve record (data not shown) among three groups. On the other hand, the homozygous group showed decreased conduction velocity when compared with the wt group in S1 cortical record ( $P < 0.05$ ), suggesting that complex gangliosides are involved in the efficient synaptic transmission, whereas they are not indispensable in peripheral nerve conductory process.

## DISCUSSION

The enrichment of gangliosides in neuronal cell membranes has suggested that they play important roles in the central nervous system (1–3). A number of studies have also suggested



**FIG. 7.** Morris water-maze performance of wt, heterozygous and homozygous mutant mice. The conditions and procedures were described in *Materials and Methods*. Twenty 12-week-old mice (10 male and 10 female) for each type were examined. (Bars = mean  $\pm$  SD.)

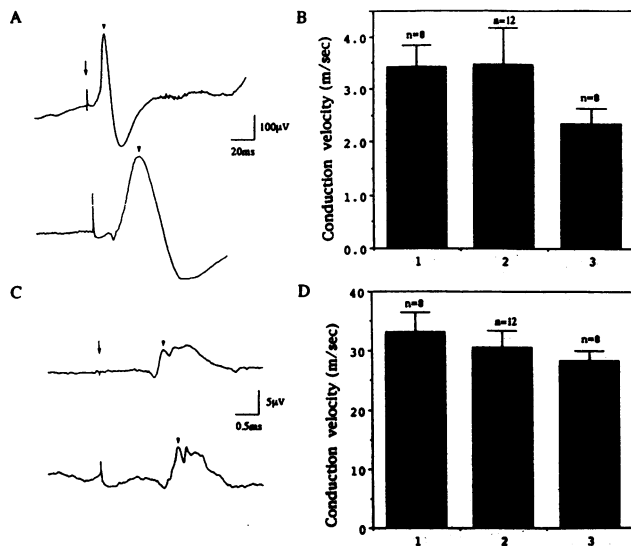


FIG. 8. S1 cortical SEP and L2 SSEP recorded from wt, heterozygote, and homozygote mice. (A) wt (upper) and homozygote mice (lower) SEP. (C) Wt (upper) and homozygote mice (lower) SSEP. Peaks are labeled according to polarity with an upward deflection being negative. Comparisons were made based on the initial peaks, because later peaks were more difficult to consistently identify in both evoked potential. Arrows indicate the stimulation points. Initial peaks are indicated by arrow heads. (B and D) Summary of the conduction velocity of SEP (B) and SSEP (D), respectively. Wt (lane 1), heterozygote (lane 2), and homozygote (lane 3) mice were examined using numbers indicated. The bars represent the mean  $\pm$  SD. A statistically significant reduction in conduction velocity was seen only in the comparison between the wild and homozygous mice in SEP ( $P < 0.05$ ). In SSEP, by L2 spinal cord recording, no significant differences among wt, heterozygous, and homozygous mice were seen.

that they are mediators for cell-cell or cell-substratum recognition, or as modulators of transmembrane signal transducers (33, 34). Consequently, gangliosides are widely believed to have essential roles in the development of the nervous system and in neural cell differentiation (1-3). Furthermore, many reports have demonstrated that exogenously added gangliosides enhanced neuritogenesis of neuronal cells *in vitro* (35-37) or that they induced regenerative responses *in vivo* (11, 38-41). However, our mutant mice lacking complex gangliosides such as GM1, GD1a, GD1b, GT1b, and GQ1b in the central nervous system showed no morphological defects in brain tissues. They were born and grew with no obvious neurological defects. Although complex gangliosides were also expected to play important roles in synaptogenesis and myelination (29-31), even electron microscopic examination revealed no apparent differences in the myelin structure or synapse formation between wt and homozygous mice. Thus, our results unexpectedly suggest that complex gangliosides enriched in brain are not as essential as widely expected. However, the decreased nerve conduction velocity in mutant mice detected in SEP suggest that gangliosides may be involved in the process of neural function, such as synaptic transmission.

The knockout mice still express GM3 and GD3 in brain tissue. Moreover, these gangliosides are expressed at much higher levels in knockout mice than in wt mice, and total amounts of brain gangliosides may not be so different between wt and homozygote mice. Therefore, it is possible that these simpler gangliosides can substitute for more complex gangliosides in whatever role gangliosides play in normal brain development. It is well known that biological systems exhibit a considerable degree of redundancy in their functional pathways and such a compensatory mechanism could be operating in these mutant mice. Mice lacking all gangliosides (e.g., GM3 synthase deficient mice) will be needed to answer this question.

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