ABCA2 Deficiency Results in Abnormal Sphingolipid Metabolism in Mouse Brain*

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ABCA2, a member of the ATP-binding cassette (ABC) transporter family, is localized mainly to late endosome/lysosomes of oligodendrocytes in brain, but the physiological role and function of ABCA2 are unknown. In this study, we generated mutant mice (ABCA2-null) by targeting the abca2 gene. ABCA2-null mice exhibited a phenotype including lower pregnancy rate and body weight, shorter latency period on the balance beam, and sensitization to environmental stress compared with wild type mice but no abnormality in the cytoarchitectonic and compact myelin structure or oligodendroglial differentiation. Lipid analysis of brain from 11 days to 64 weeks of age revealed significant accumulation of gangliosides along with reduced sphingomyelin (SM) from 4 weeks to 64 weeks of age and accumulation of cerebrosides and sulfatides at 64 weeks of age in ABCA2-null mice compared with wild type mice. In addition, a significant accumulation of the major ganglioside GM1 and reduced SM was detected in the myelin fraction of ABCA2-null brain. Comparison of ABCA2-null and wild type mice revealed weak ABCA2 immunoreactivity in some large pyramidal cells of wild type brain. These results suggest that ABCA2 is involved in the intracellular metabolism of sphingolipids in the brain, particularly SM and gangliosides in oligodendrocytes and certain neurons.

ATP-binding cassette (ABC)3 transporters are involved in ATP-dependent transport of various substrates, such as ions, peptides, and lipids, across membranes (1, 2). Among members of the A subfamily, ABCA1 has been shown to be involved in phospholipid efflux by binding to apoA-I (12, 13), suggesting involvement of the A subfamily in lipid transport linked to myelination processes in matured myelinating oligodendrocytes (16). However, there is no evidence demonstrating the function of ABCA2 as a lipid transporter in myelination processes. In this study, we generated an ABCA2-null mouse line by gene targeting in embryonic stem (ES) cells to clarify the physiological role and function of ABCA2 in the brain.

EXPERIMENTAL PROCEDURES

Generation of ABCA2-null Mice—The abca2 gene fragment was isolated from a 129Sv mouse λ-bacteriophage genomic library (Stratagene). The targeting vector for deletion of exons 13–22 of the murine abca2 gene was constructed as follows. A 7.0-kb EcoRI and a 3.1-kb XhoI–SalI fragment were used as the 5’ and 3’ arm, respectively. These arms were inserted on either side of the phosphoglycerate kinase-neo cassette containing the neomycin phosphotransferase gene (neo) under control of the mouse phosphoglycerate kinase promoter, in pBluescript (see Fig. 1A). External to the 3’ region of homology with the target locus, a negatively selectable marker gene (the herpes simplex virus thymidine kinase gene) was placed under the control of the MCI promoter.

The targeting vector was electroporated into RW4 ES cells, and the G418-resistant clones were screened by Southern blot analysis of ES cell genomic DNA (17), which was digested with HindIII followed by hybridization with an external 3’ probe (2.2 kb). Mutant ES cells were microinjected into blastocysts of C57BL/6J females. The resulting chimeras were used to gener-
ate heterozygous and subsequently homozygous mutant offspring against the C57BL/6J genetic background.

The mice were genotyped for the introduced abca2 gene mutation by PCR analysis of tail genomic DNA using the ABCA2–5′ (5′-GATCTTCCAGACAGGGAGG-3′) and ABCA2–3′ primers (5′-AGACGAGGCGATAGAGGAG-3′) for wild type and ABCA2–5′ and Neo-3′ primers (5′-CGAAGGAGAACTGCTATTG-3′) for knock-out. Disruption of the abca2 gene was confirmed by Southern and immunoblot analyses (16).

The mice used in the present study were of mixed background (C57BL/6J and 129 SVJ), and littermate controls were used for all experiments. All experiments involving animal use were approved by the Akita University Institute Committee for Animal Studies. The abbreviations for wild type, heterozygous, and homozygous mutant mice are wild type (WT) or +/+ or −/−, and ABCA2-null or −/−, respectively.

Behavior of Mice—Behavioral tests, except for environmental stress, were videotaped and scored at a later time by an experimenter blind to their genotype.

For balance beam analysis, each mouse was placed at the center and perpendicular to a rounded plastic beam (60 cm length, 1 cm diameter) suspended 60 cm above a foam pillow. The maximum latency to fall (up to 75 min) was recorded in five consecutive trials. For analysis of hind limb reflex extension, each mouse was suspended by the tail for 5 s.

To investigate the response to environmental stress, 6–9 mice at 4–16 weeks of age (body weight, 10–33 g) were moved onto wet sawdust (25 g of sawdust in 250 ml of water) in the mice at 4–16 weeks of age (body weight, 10–33 g) were moved onto wet sawdust (25 g of sawdust in 250 ml of water) in the home cage (bottom size, 18 × 28 cm) for 24 h at 25 °C. Each mouse was then bred normally in a home cage for 1 week, and viability was determined.

Immunoblot Analysis—Immunoblotting was performed as previously described with the following antibodies: anti-ABCA1 (1:5000, generously provided by Dr. Kazumitsu Ueda, Kyoto University and Kyowa Hakko Co., Ltd.) (18), anti-ABCA2 (1:1000) (16), anti-ABCA3 (1:1000) (19), anti-ABCA4 (1:1000, Santa Cruz Biotechnology), anti-ABCA7 (1:50, generously provided by Dr. Shinji Yokoyama, Nagoya City University) (20), myelin-associated glycoprotein (anti-MAG) (1:3000, Chemicon) (21), anti-myelin basic protein (anti-MBP) (1:20, Dako) (21), anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (anti-CNPase) (1:1000, Sigma) (21), or anti-β-tubulin antibody (1:5000, Sigma).

Histological Analysis—Animals were deeply anesthetized with urethane and perfused with 4% paraformaldehyde. The brain was removed, post-fixed, and sectioned coronally. Sections were stained by standard cresyl violet procedure (22) to examine cytarchitectonic structures. Immunohistochemical staining was performed by the peroxidase-anti-peroxidase method as described previously (16). The primary antibody used was anti-ABCA2 (1:2000) or anti-galactose-binding protein (anti-FGP) (1:2000, Dako). The sections were mounted on glass slides and then counterstained by cresyl violet. For electron microscopic analysis, the mice were perfused with 2% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer (pH 7.4), and a posterior part of the corpus callosum and a tractus pyramidalis anterior part of the cervical spinal cord (C4) was dissected to expose the cross-sectional area. The samples were further fixed in 1% osmium tetroxide in cacodylate buffer at room temperature for 2 h, dehydrated in a graded series of ethanol (70–100%), and embedded in EPON 812 resin. Ultrathin sections were cut in the transverse plane with an ultramicrotome (LKB 8800 Ultratome III; Bromma), stained with uranyl acetate and lead citrate, and observed with an electron microscope at 80 kV (Hitachi, H-7650).

Isolation of Myelin Fractions—Brain was homogenized 10 strokes with a Dounce homogenizer in 0.32 M sucrose. The myelin fractions were isolated as described previously (23) and stored at −80 °C until use.

Extraction of Total Lipids and Isolation of Gangliosides—Total lipids in brain tissue and the isolated myelin fractions were extracted by chloroform:methanol (2:1, v/v) and chloroform:methanol:water (1:2.0.8, v/v) (24). For purification of gangliosides, total lipids were applied to a DEAE-Sephadex A-25 column (Sigma) equilibrated with chloroform:methanol:water (30:60:8, v/v). Although neutral lipids passed through the column, acid lipids were retained on the column and recovered by elution with chloroform:methanol:0.8 M sodium acetate (pH 7.0) (30:60:8, v/v) (25). To hydrolyze coexisting phospholipids, acidic lipids were incubated in 0.2 M methanolic NaOH at 37 °C for 2 h followed by neutralization with acetic acid. Purified gangliosides were obtained by desalting the neutralized sample using a Sep-Pak C18 cartridge (Millipore).

Chromatographic Separations—For separation of each ganglioside, aliquots of the purified gangliosides were developed on silica gel 60 high-performance thin-layer chromatography plates (Merck, 10 × 20 cm) with appropriate standards, using chloroform:methanol:0.2% CaCl2 in water (55:45:10, v/v). For the separation of cerebrosides, each phospholipid, and sulphatides, aliquots of total lipids were developed on silica gel 60 thin-layer chromatography plates (Merck, 20 × 20 cm) using two-dimensional solvent systems; the first and the second chromatographic runs were performed with chloroform:methanol:7 M ammonia (65:30:4, v/v) and with chloroform:methanol:lactic acid:water (170:25:25:4, v/v) at 90° to the original direction (26). After development, the plates were sprayed with primuline (0.001% w/v in acetone:water (4:1)) and viewed under UV light (365 nm) for the localization of separated compounds. Each lipid spot on the high-performance and thin-layer chromatography plates was scraped off using a razor, and the spots of cerebrosides, each phospholipid, and sulphatides were extracted from silica gel by chloroform:methanol:water (1:2:0.8, v/v).

Lipid Analysis—The amount of cholesterol contained in total lipids was measured using an enzymatic assay kit (Kyowa Medex). For measurement of the amount of inorganic phosphate in the phospholipids, individual phospholipids extracted from silica gel were digested in perchloric acid (70%) for 90 min at 200 °C. The inorganic phosphate was quantified colorimetrically (27). The calibration curves were prepared using potassium dihydrogen phosphate as the standard.

For measurement of the amount of sphingoid base in cerebrosides and sulphatides, the lipids were extracted from silica gel and hydrolyzed by aqueous methanolic HCl reagent (concentrated hydrochloric acid:water:methanol (8.6:9.4:82) v/v) for 18 h at 70 °C and then quantified colorimetrically by the methyl orange method using N-palmitoyl-D-sphingosine for calibration (28). For measurement of the amount of sialic acid in the gangliosides, individual gangliosides scraped from high-perfor-
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mance thin-layer chromatography plates were suspended in water and reacted with resorcinol-HCl reagent for 1 h at 100 °C (29). The sialic acid was quantified colorimetrically as described previously (30). The calibration curves were prepared using N-acetylneuraminic acid as the standard.

Statistics—Data normalized by the protein content for lipid analyses were represented by means ± S.D.; data obtained by electron microscopic and behavior analysis were represented by means ± S.E. Statistical analysis was performed by the two-tailed t test or χ²-test.

RESULTS

Generation of ABCA2-null Mice—To investigate the physiological function of ABCA2, ABCA2-null mice were generated by deleting exons 13–22 containing the sequence encoding the Walker A motif of the nucleotide binding domain 1 (31), which is essential for the function of ABC transporter proteins (Fig. 1A). Several correctly targeted ES cell clones were isolated and injected into blastocysts, which developed germ line transmission. Southern blotting of tail genomic DNA with a 3’ probe outside the targeting vector showed the predicted HindIII DNA fragment sizes of 5.7 and 7.3 kb for the WT and the targeted allele, respectively (Fig. 1B). PCR analysis showed the predicted DNA fragment sizes of 161 and 449 bp for the WT and the targeted allele, respectively (Fig. 1C). Subsequent immunoblotting analysis demonstrated the complete loss of detectable ABCA2 protein (260 kDa) in homozygous mutant mice from 11 days to 64 weeks (Fig. 1D), confirming successful targeting of ABCA2. In addition, the expression levels of other A subfamily members in brain, including ABCA1, ABCA3, ABCA4, and ABCA7, were not altered by ABCA2 deficiency (Fig. 1D).

Phenotype of ABCA2-deficient Mice—Heterozygous crosses showed that the distribution of the three genotypes (+/+ , +/− , −/−) was according to Mendelian inheritance (265:508:220) and that there was no change in the gender ratio of ABCA2-null mice (male/female, 111/109), indicating no reduction in embryonal viability. Both male and female ABCA2-null mice survived for >1 year and were fertile. Homozygous crosses for 1 week exhibited a pregnancy rate of female ABCA2-null mice (38.6%, n = 83) that was lower than those of WT mice (68.1%, n = 69, p < 0.001). The body weights of male and female ABCA2-null mice were lower than those of WT mice at 5 and 10 weeks (Fig. 2A). This difference in body weight was also observed at 18 weeks (data not shown).

When hung vertically with their tails upward, ABCA2-null mice usually extended their limbs downward in a manner similar to the parachute reflex in human infants but with their hind limb shaking. At 4–5 weeks, almost all of the ABCA2-null and much fewer WT mice showed this phenotype (Table 1). In addition, the maximum latency period of ABCA2-null mice on the balance beam was shorter than that of WT mice at 18 weeks (Fig. 2B).

FIGURE 1. Targeted disruption of the abca2 gene and generation of ABCA2-null mice. A, scheme of targeted disruption of murine abca2 by homologous recombination. E, EcoRI; H, HindIII; S, SphI; X, XbaI; WA, Walker A; WB, Walker B. To confirm deletion of abca2 in mutant mice, Southern blot analysis of HindIII-digested tail genomic DNA was performed with an external 3’ probe as noted in A. Expected fragment sizes are 5.7 kb for WT and 7.3 kb for mutant ABCA2. C, to genotype the mutant mice, PCR analysis of tail genomic DNA was performed with specific primers. Expected product sizes were 161 bp for WT and 449 bp for mutant ABCA2. D, immunoblot analysis shows the patterns of ABCA1, ABCA2, ABCA3, ABCA4, and ABCA7 in WT and ABCA2-null brain from 11 days to 64 weeks.

FIGURE 2. Phenotype of ABCA2-null mice. The body weight (A) and maximum latency period on the balance beam (B) of WT (open bars) and ABCA2-null mice (closed bars) are shown. The values are means ± S.D.
Because the ABCA2-null mice appeared to be sensitized to external factors such as sound and body touch, the response to stress was evaluated. ABCA2-null and WT mice at 4–16 weeks were moved onto wet sawdust in the home cage for 24 h. The death rate of ABCA2-null mice after 1 week was much higher than that of WT mice (Table 2). This result was independent of body weight and age (data not shown).

Histological Analysis and Maturation of Oligodendrocytes in ABCA2-null Mice—To investigate the effect of ABCA2 deficiency on brain, in which ABCA2 mRNA is most abundant (14), we observed the cytoarchitectonic structure and astrocytic distribution at 8 weeks. Cresyl violet staining of ABCA2-null brain showed that the cytoarchitectonic structure of regions such as the cortex and hippocampus was indistinguishable from that of WT brain (Fig. 3A and data not shown). Immunohistochemical staining with an anti-GFAP antibody showed normal astrocytic distribution in the cerebrum of ABCA2-null brain (Fig. 3B). These data indicate that ABCA2 deficiency does not affect the cytoarchitectonic structure of the brain.

In a previous study, we demonstrated that the temporal and spatial profiles of ABCA2-positive oligodendrocytes in the developing brain coincide well with those of myelin formation, suggesting a role of ABCA2 in lipid transport associated with myelination processes (15, 16). To clarify the effect of ABCA2 disruption on myelin formation, we compared the ultrastructure of compact myelin and axons of WT and ABCA2-null brain at 12 weeks, when the myelination process has largely been completed in brain tissue (32). Analysis of the corpus callosum showed no morphological difference between WT and ABCA2-null brain (Fig. 3C). The axonal and fiber diameter of myelinated fibers, the ratio of fiber area to axonal area of myelinated fibers, and the ratio of unmyelinated fibers to myelinated fibers in ABCA2-null brain were not significantly different from those in WT brain (Table 3). In spinal cord, the axonal and fiber diameter of myelinated fibers in ABCA2-null brain (0.86 ± 0.02 and 1.25 ± 0.03 μm (n = 386), respectively) were also similar to those of WT brain (0.89 ± 0.02 and 1.30 ± 0.03 μm (n = 325)). In addition, immunoblot analysis of myelin proteins revealed that the levels of myelin-associated glycoprotein, myelin basic protein, and CNPase in ABCA2-null brain were similar to those in WT brain from 11 days to 64 weeks (Fig. 3D). These data indicate that there is no difference in the formation of compact myelin structure or oligodendroglial differentiation and stability during aging between WT and ABCA2-null brain.

Lipid Analysis of ABCA2-null Brain—Because members of the ABC transporter A subfamily are proposed to function as lipid transporters, we compared the lipid content of WT and ABCA2-null mice. Alterations of major lipid contents, including cholesterol, phospholipids, cerebrosides, sulfatides, and gangliosides, were investigated at 11 days and 4, 16, 32, and 64 weeks in these mice. Each of these ages is important in the rodent: 11 days is the infant stage at which the expression of ABCA2 protein increases in brain (16), 4 weeks is the growth stage when spatial distribution of ABCA2-positive oligodendrocytes is completed (16), 16 weeks is the young adult stage (8–24 weeks), 32 weeks is the middle adult stage (24–56 weeks), and 64 weeks is the old adult stage (>56 weeks).

The contents of cholesterol and phospholipids, which are known to be substrates for both ABCA1 and ABCA7 transporters, were then analyzed. The amounts of chole-

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**TABLE 1**

| Gender | Genotype | Shaking | Total test | p value |
|--------|----------|---------|------------|---------|
| Male   | WT       | 4       | 58         | <0.001  |
| KO*    | WT       | 42      | 44         |         |
| Female | WT       | 6       | 54         | <0.001  |
| KO     | WT       | 60      | 61         |         |

* KO, knock-out.

**TABLE 2**

| Gender | Genotype | Survival | Total test | p value |
|--------|----------|----------|------------|---------|
| Male   | WT       | 17       | 18         | <0.01   |
| KO*    | WT       | 13       | 24         |         |
| Female | WT       | 35       | 39         | <0.001  |
| KO     | WT       | 6        | 31         |         |

* KO, knock-out.

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**FIGURE 3.** Histological and immunoblot analysis of brain. A. cresyl violet staining of sections of brain prepared from WT (left panel) and ABCA2-null (right panel) mice at 8 weeks. CC, corpus callosum; HP, hippocampus; GM, gray matter; WM, white matter. Scale bar, 1 mm. B, astrocytic distribution by immunohistochemical staining of glial fibrillary acidic protein is shown. Brain sections were prepared from WT (left panel) and ABCA2-null (right panel) mice at 8 weeks. Scale bar, 0.2 mm. C, electron microscopic images were prepared from corpus callosum of WT (left panel) and ABCA2-null (right panel) brain at 12 weeks. Scale bar, 0.5 μm. The images are quantified in Table 1. D, immunoblot analysis shows the patterns of myelin-associated glycoprotein (MAG), myelin basic protein (MBP), CNPase, and β-tubulin of WT and ABCA2-null brain from 11 days to 64 weeks. β-Tubulin was used as the loading control.
terol and total phospholipids in ABCA2-null brain were similar to those in WT brain from 11 days to 64 weeks (Fig. 4A and Table 4). As shown in Table 4, in phospholipid species, the amount of phosphatidylcholine and phosphatidylserine was decreased to 0.86- and 0.85-fold at 4 weeks, respectively, and the amount of phosphatidylethanolamine was increased to 1.05-fold at 32 weeks, but these alterations were slight and transient. On the other hand, the level of sphingomyelin (SM) was constantly decreased. Thus, ABCA2 deficiency causes a constant reduction of SM in brain after the growth stage but does not affect the cholesterol and glycerophospholipid levels.

We also investigated the contents of glycosphingolipids such as galactosylceramides, sulfatides, and gangliosides, which are abundant in brain compared with other tissues. The amounts of cerebrosides, which consist mainly of galactosylceramides, and sulfatides in ABCA2-null mice brain were similar to those in WT brain from 11 days to 32 weeks and increased to 1.16- and 1.60-fold only at 64 weeks, respectively. On the other hand, the level of sphingomyelin (SM) was significantly increased to 2.11-fold (Fig. 5C), and phosphatidylethanolamine (PE), phosphatidylserine (PS), and total phospholipid levels were slightly decreased to 0.87-, 0.81-, and 0.86-fold, respectively (Fig. 5B). As observed in whole brain, SM content was decreased to 0.60-fold (Fig. 5B), whereas GM1 content was increased to 2.11-fold (Fig. 5E), suggesting ABCA2 involvement in SM and GM1 metabolism in the myelin component. Cholesterol, cerebroside, and sulfatide contents of the myelin fractions of ABCA2-null brain at 32 weeks were similar to those of WT brain (Fig. 5, A, C, and D), and phosphatidylethanolamine (PE), phosphatidylserine (PS), and total phospholipid levels were slightly decreased to 0.87-, 0.81-, and 0.86-fold, respectively (Fig. 5B). As observed in whole brain, SM content was decreased to 0.60-fold (Fig. 5B), whereas GM1 content was increased to 2.11-fold (Fig. 5E), suggesting ABCA2 involvement in SM and GM1 metabolism in the myelin component of oligodendrocytes. GD1a, GT1a, GT1b, and GQ1b, which are known to be minor gangliosides in the myelin component after the young adult stage (33),

**TABLE 3**

Electron microscopic analysis of WT and ABCA2-null myelin

|          | WT                  | ABCA2-null           |
|----------|---------------------|----------------------|
| Axon (µm)| 0.66 ± 0.01 (n = 870) | 0.66 ± 0.01 (n = 829) |
| Fiber (µm)| 0.69 ± 0.01 (n = 869) | 0.69 ± 0.01 (n = 819) |
| Fiber/axon| 2.22 ± 0.02 (n = 857) | 2.22 ± 0.02 (n = 814) |
| Unmyelinated/myelinated| 2.07 ± 0.22 (n = 4) | 1.93 ± 0.33 (n = 4) |

**FIGURE 4. Lipid analysis of whole brain.** The contents of cholesterol (A), cerebrosides (B), and sulfatides (C) in WT (open bars) and ABCA2-null (closed bars) brain from 11 days to 64 weeks were measured. The values are the means ± S.D. of three independent experiments.

**TABLE 4**

Phospholipid contents in WT and ABCA2-null brain

| Age     | Genotype | PC (nmol/mg protein) | PE (nmol/mg protein) | PI (nmol/mg protein) | PS (nmol/mg protein) | PA (nmol/mg protein) | CL (nmol/mg protein) | SM (nmol/mg protein) | Total (nmol/mg protein) |
|---------|----------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|------------------------|
| 11 days | +/-      | 272.5 ± 3.5          | 154.5 ± 25.7         | 17.2 ± 1.7           | 43.6 ± 1.0           | ND\(^b\)             | 4.1 ± 0.2            | 9.8 ± 0.9            | 501.7 ± 28.8           |
|         | +/-      | 281.8 ± 6.4          | 157.1 ± 10.9         | 20.3 ± 6.6           | 40.5 ± 2.1           | ND\(^b\)             | 3.6 ± 1.1            | 9.7 ± 2.7            | 513.0 ± 12.3           |
| 4 weeks | +/+      | 304.0 ± 15.7         | 254.4 ± 13.0         | 23.2 ± 1.5           | 71.6 ± 2.2           | 6.2 ± 1.7            | 21.1 ± 1.6           | 653.4 ± 31.1          |
|         | +/-      | 296.8 ± 8.1          | 241.4 ± 17.6         | 19.9 ± 1.7\(^c\)    | 61.1 ± 5.5\(^b\)    | 8.3 ± 1.5\(^b\)     | 22.1 ± 1.6\(^b\)     | 634.3 ± 35.1\(^b\)   |
| 16 weeks| +/-      | 326.6 ± 15.5         | 321.4 ± 18.7         | 25.1 ± 0.9           | 93.2 ± 7.9           | 12.2 ± 2.0           | 10.9 ± 3.5           | 35.4 ± 2.3            | 824.8 ± 49.6           |
|         | +/-      | 337.5 ± 11.4         | 336.5 ± 14.6         | 26.9 ± 2.0           | 100.7 ± 2.0          | 13.9 ± 0.8           | 9.1 ± 0.1            | 28.9 ± 2.2            | 853.5 ± 28.5           |
| 32 weeks| +/-      | 317.7 ± 12.9         | 310.2 ± 4.4          | 29.0 ± 0.7           | 104.9 ± 2.9          | 14.3 ± 2.2           | 6.6 ± 0.7            | 33.7 ± 2.6            | 871.0 ± 23.2           |
|         | +/-      | 327.4 ± 6.4          | 326.9 ± 3.4\(^d\)    | 29.0 ± 4.2           | 108.4 ± 3.1          | 13.5 ± 2.3           | 7.2 ± 0.2            | 26.4 ± 0.9            | 839.1 ± 13.1           |
| 64 weeks| +/-      | 349.4 ± 12.6         | 374.7 ± 17.2         | 29.1 ± 2.4           | 121.4 ± 8.0          | 18.5 ± 1.7           | 9.5 ± 0.4            | 38.4 ± 0.8            | 940.9 ± 40.0           |
|         | +/-      | 349.2 ± 24.2         | 384.9 ± 32.8         | 27.9 ± 2.2           | 120.3 ± 10.8         | 15.1 ± 7.6           | 6.8 ± 2.2            | 30.1 ± 2.7\(^c\)     | 934.2 ± 79.4           |

a Values represent means ± S.D. of three independent experiments. Data in parentheses indicate the ratio of +/- to +/- +. Abbreviations for phospholipids are shown as follows: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; CL, cardiolipin; SM, sphingomyelin.  
\(^b\) ND, not detected.  
\(^c\) Significantly different from control values at \(p < 0.05\).  
\(^d\) Significantly different from control values at \(p < 0.01\).
TABLE 5
Ganglioside contents in WT and ABCA2-null brain

| Age     | Genotype | Concentrationsa |
|---------|----------|-----------------|
|         | GM1      | GD1a            | GT1a            | GD1b      | GT1b      | GQ1b      | Total    |
| 11 days | +/+      | 0.68 ± 0.08     | 3.24 ± 0.11     | 0.17 ± 0.01 | 0.32 ± 0.03 | 2.17 ± 0.07 | 0.18 ± 0.01 | 6.76 ± 0.19 |
|         | −/−      | 0.61 ± 0.06     | 3.47 ± 0.23     | 0.19 ± 0.02 | 0.23 ± 0.03 | 2.31 ± 0.06 | 0.22 ± 0.02 | 6.03 ± 0.31 |
| 4 weeks | +/+      | 1.25 ± 0.09     | 3.63 ± 0.20     | 0.20 ± 0.04 | 0.51 ± 0.03 | 2.34 ± 0.14 | 0.41 ± 0.04 | 8.35 ± 0.42 |
|         | −/−      | 1.31 ± 0.16     | 4.23 ± 0.19b    | 0.32 ± 0.04b | 0.49 ± 0.04 | 3.37 ± 0.09d | 0.82 ± 0.03d | 10.54 ± 0.47d |
|         |          | (1.04)          | (1.17)          | (1.08)     | (0.73)     | (1.07)     | (1.21)    | (1.04)    |
| 16 weeks| +/+      | 1.88 ± 0.11     | 3.31 ± 0.11     | 0.26 ± 0.01 | 0.61 ± 0.02 | 3.18 ± 0.05 | 0.77 ± 0.02 | 10.02 ± 0.24 |
|         | −/−      | 3.05 ± 0.16d    | 4.04 ± 0.29abd  | 0.46 ± 0.02abd | 0.51 ± 0.10 | 4.30 ± 0.51ab | 1.55 ± 0.21ab | 13.92 ± 1.19ab |
|         |          | (1.62)          | (1.22)          | (1.75)     | (0.83)     | (1.35)     | (2.01)    | (1.39)    |
| 32 weeks| +/+      | 2.02 ± 0.25     | 2.53 ± 0.49     | 0.27 ± 0.05 | 0.49 ± 0.08 | 2.51 ± 0.37 | 0.74 ± 0.15 | 8.56 ± 1.28 |
|         | −/−      | 3.72 ± 0.50d    | 2.82 ± 0.40     | 0.41 ± 0.05b | 0.38 ± 0.05 | 3.03 ± 0.24 | 1.18 ± 0.09bd | 11.54 ± 1.27bd |
|         |          | (1.84)          | (1.11)          | (1.56)     | (0.78)     | (1.20)     | (1.59)    | (1.35)    |
| 64 weeks| +/+      | 1.83 ± 0.37d    | 2.02 ± 0.25     | 0.15 ± 0.03 | 0.58 ± 0.15 | 2.52 ± 0.08 | 0.61 ± 0.03 | 7.72 ± 0.56 |
|         | −/−      | 6.04 ± 0.39bd   | 3.21 ± 0.33bd   | 0.43 ± 0.03bd | 0.52 ± 0.02 | 4.05 ± 0.21bd | 1.51 ± 0.12bd | 15.75 ± 0.93bd |
|         |          | (3.29)          | (1.58)          | (2.90)     | (0.89)     | (1.60)     | (2.47)    | (2.04)    |

a Values represent means ± S.D. of three independent experiments. Data in parentheses indicate the ratio of −/− to +/+.
b Significantly different from control values at p < 0.05.
c Significantly different from control values at p < 0.005.
d Significantly different from control values at p < 0.01.

FIGURE 5. Lipid analysis of myelin. The contents of cholesterol (A), each phospholipid and total phospholipids (B), cerebrosides (C), sulfatides (D), and GM1 (E) in myelin fractions of WT (open bars) and ABCA2-null (closed bars) brain at 32 weeks were measured. The values are the means ± S.D. of three independent experiments.

FIGURE 6. ABCA2 expression in oligodendrocytes and some neurons of WT brain. Immunostaining images of ABCA2 in the somatosensory cortex of WT brain at 8 weeks are shown. Arrowheads indicate oligodendrocytes. The arrow and asterisk in Layer V indicate a pyramidal cell and satellite oligodendrocyte, respectively. Scale bars, 10 μm.

were present at a trace amount in myelin fractions of both WT and ABCA2-null brain (data not shown).

ABCA2 Expression in WT Neurons—We previously proposed that ABCA2 is localized mainly in oligodendrocytes and Schwann cells by immunohistochemical studies using anti-ABCA2 antibody (14–16, 34, 35). In the present study, establishment of the ABCA2-null mouse has enabled us to evaluate the expression of ABCA2 by immunohistochemical investigation. In addition to oligodendrocytes, ABCA2 immunoreactivity in neurons was detected by 4 weeks (the growth stage) in the cerebral cortex of WT brain but much more weakly than in oligodendrocytes (Fig. 6 for 8 weeks data, not shown for other ages). ABCA2 expression in some large pyramidal cells in layer V, presumably cortical output neurons, was higher than that in the neurons of other layers. We confirmed that such granular ABCA2 immunoreactivity was not detected in neurons and oligodendrocytes in ABCA2-null mice (data not shown).

DISCUSSION

In the present study, we have demonstrated that disruption of the abca2 gene results in alterations of sphingolipid metabolism in brain, a constant reduction of SM and the accumulation of glycosphingolipids with no apparent histological abnormalities. In ABCA2-null brain, gangliosides including GM1,
GD1a, GT1a, GT1b, and GQ1b age-dependently accumulated after 4 weeks (the growth stage), except for GM1, which accumulated from 16 weeks (the young adult stage) (Table 5), whereas cerebroside and sulfatide levels increased only at 64 weeks (the old adult stage) (Fig. 4, B and C). In contrast, the SM level was constantly decreased after 4 weeks (the growth stage) in ABCA2-null brain (Table 4). These results suggest a more important role of ABCA2 in the metabolism of SM and gangliosides than in cerebrosides and sulfatides. Similarly, in the myelin fraction of ABCA2-null brain, the SM content decreased, whereas GM1 (but not cerebrosides or sulfatides) accumulated as in WT brain (Fig. 5), although both cerebrosides and sulfatides were highly enriched in oligodendrocytes, suggesting that ABCA2 also is involved in the metabolism of SM and GM1 (one of the ganglioside species) in the myelin component of oligodendrocytes.

Although other ganglioside species besides GM1, including GD1a, GT1a, GT1b, and GQ1b, accumulated in ABCA2-null brain, these ganglioside species were present at trace amounts in myelin fractions of both WT and ABCA2-null brain (data not shown), as previously described in mice after the young adult stage (33). In the present study, we confirmed weak ABCA2 expression in some large pyramidal cells (Fig. 6) by comparison of WT and ABCA2-null mice. In fact, it recently was suggested that ABCA2 is expressed in early neural progenitors in vitro and in a subset of GABAergic and glutamatergic neurons in vivo (36). These results suggest that the accumulation of ganglioside species other than GM1 in the ABCA2-null brain after the young adult stage is due to alterations of ganglioside levels in components of oligodendrocytes and neurons other than myelin.

Abnormal metabolism of sphingolipid, especially SM and gangliosides, in ABCA2-null brain might be due to perturbation of sphingolipid synthesis and/or degradation. SM and gangliosides are synthesized from ceramide, the common precursor of all classes of sphingolipids, in the lumen of the Golgi. Ceramide is flipped from the cytosolic side of the Golgi to the lumen side, where SM is synthesized by the addition of phosphocholine to ceramide. Alternatively, ceramide is converted to glucosylceramide on the cytosolic side of the Golgi and then glucosylceramide is flipped to the lumen side, where gangliosides are synthesized by the addition of sugars to glucosylceramide. ABCA2 is localized not only to late endosome/lysosomes but also to Golgi compartments (15). Thus, ABCA2 might be involved in the metabolism of SM and gangliosides in the synthetic pathway from ceramide. Alternatively, SM is converted to ceramide by acidic SMase in lysosomal compartments, whereas gangliosides including GD1a, GT1a, GD1b, GT1b, and GQ1b are converted to GM1 ganglioside by sialidase, upon which GM1 is converted to GM2 and GA1 by GM1-β-galactosidase and sialidase, respectively. Thus, deficiency of ABCA2 at the lysosomal membrane could result in the abnormal metabolism of sphingolipids in the degradation pathway. Further investigation is required to determine the function of ABCA2.

Deficiency of GM1-β-galactosidase results in the accumulation of a large amount of GM1, referred to as gangliosidosis (37–39). Mice having GM1 gangliosidosis show considerable accumulation of GM1 (4.12-fold) at 10 weeks (the young adult stage) compared with WT and also clinical phenotypes such as tremor, ataxia, and abnormal gait beyond 20 weeks (37, 39–40). On the other hand, ABCA2-null mice show considerably less accumulation of GM1 (3.29-fold) even at 64 weeks (the old adult stage) and no such severe phenotype. The lesser accumulation of GM1 and the smaller alterations of other sphingolipids in ABCA2-null mice may underlie the milder phenotype, including lower pregnancy rate and body weight, a shorter latency period on the balance beam, and sensitization to environmental stress.

There is a very recent report that ABCA2 knock-out mice show a mild phenotype including tremor and hyperactivity, similar to the hind limb shaking (Table 1) and other hyperactivity (data not shown) of our ABCA2-null mice (41). However, that study found abnormal myelin structure despite the lack of change in lipid composition in the brain. They analyzed lipid content in brain using liquid chromatography/mass spectrometry. Liquid chromatography/mass spectrometry is, in general, a high-sensitivity method. However, its sensitivity greatly depends on the efficiency of ionization of compounds. Indeed, we could not find any difference in the quantity of SM (of which ionization considerably varies) between ABCA2-null and WT brain by liquid chromatography/mass spectrometry (data not shown). Regarding myelin structure, abnormal myelin structure was found in cerebrum and spinal cord, but the precise region and age are not shown in their paper. We analyzed myelin structure in specific regions, a posterior part of the corpus callosum (splenium of corpus callosum) and a tractus pyramidalis anterior part of the cervical spinal cord (C4) of the young adult stage, as myelin size can vary greatly in different regions. Unexpectedly, we could not find any alterations of myelin structure in ABCA2-null mice. We conclude that the alteration of sphingolipid levels in ABCA2-null brain is insufficient to affect myelin structure in these regions at the young adult stage. The discrepancies between their results and ours may be due to a different targeting approach, a different analytical method, or other unknown factors.

In summary, the present study shows that ABCA2 deficiency causes abnormalities in the metabolism of sphingolipids (especially SM and gangliosides) in brain, suggesting a role of ABCA2 in the metabolism of these lipids. Further study is required to clarify the function of ABCA2 in brain lipid metabolism in more detail.

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