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Biochemistry and Neuropathology of Mice Doubly Deficient in Synthesis and Degradation of Galactosylceramide

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We have generated mice doubly deficient in both synthesis and degradation of galactosylceramide by crossbreeding twitcher mice and galactosylceramide synthase (UDP-galactose:ceramide galactosyltransferase, CGT) knockout mice. The prediction that the phenotype of the doubly deficient mice should be the same as the *cgt* $^{-/-}$ mice, since the degrading enzyme should not be necessary if the substrate is not synthesized, proved to be only partially correct. In early stages of the disease, the doubly deficient mice (*galc* $^{-/-}$, *cgt* $^{-/-}$) were essentially indistinguishable from the *cgt* $^{-/-}$ mice. However, the doubly deficient mice had a much shorter life span than *cgt* $^{-/-}$ mice. Both galactosylceramide and galactosylsphingosine (psychosine), were undetectable in the brain of the *cgt* $^{-/-}$ and the doubly deficient mice. The characteristic twitcher pathology was never seen in the *galc* $^{-/-}$, *cgt* $^{-/-}$ mice. However, after 43 days, neuronal pathology was observed in the brainstem and spinal cord. This late neuronal pathology has not been seen in the CGT knockout mice but has been described in some long surviving bone marrow-transplanted twitcher mice. Furthermore, the motor segment of the trigeminal nerve of the *galc* $^{-/-}$, *cgt* $^{-/-}$ mice showed severe degeneration not seen in either twitcher or CGT knockout mice. Thus, the *galc* $^{-/-}$, *cgt* $^{-/-}$ mice, while primarily showing the *cgt* $^{-/-}$ phenotype as predicted, develop late pathology that is seen only in twitcher mouse and also a unique pathology in the trigeminal nerve. These observations indicate that the functional relationship between galactosylceramidase and galactosylceramide synthase is complex. J. Neurosci. Res. 59:170–178, 2000. © 2000 Wiley-Liss, Inc.

Key words: galactosylceramidase; galactosylceramide synthase; double knockout; twitcher

Galactosylceramide, together with its sulfate ester, sulfatide, is the most characteristic lipid in the myelin sheath, constituting more than 20% of its dry weight. More than 95% of total galactosylceramide in the body is present as a myelin constituent. While its precise physiological function in the myelin sheath remains largely a matter of conjecture, perturbation of its metabolism causes serious brain dysfunction. A lysosomal enzyme, galactosylceramidase (GALC), is responsible for physiological degradation of galactosylceramide (Bowen and Radin, 1969) and its genetic defect results in one of the classical genetic leukodystrophies, Krabbe disease (globoid cell leukodystrophy) in humans and equivalent disorders in several mammalian species, most notably the twitcher mutant in the mouse (Kobayashi et al., 1980; Suzuki et al., 1995). On the other hand, UDP-galactose: ceramide galactosyltransferase (CGT) synthesizes galactosylceramide with either α-hydroxylated or nonhydroxylated fatty acids. No

In this article, the genes encoding galactosylceramidase and galactosylceramide synthase are written with lower-case italic letters (*galc*, *cgt*) and their products (enzyme proteins) with capital letters (GALC, CGT). Although the twitcher mutation should strictly be expressed as *galc* $^{twi/twi}$, the designation of *galc* $^{-/-}$ is used to denote the twitcher mutant, since there is no danger of confusion.

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spontaneous CGT mutant is known (Morell and Radin, 1969). Upon its recent cloning, we, as well as the group of Stoffel, utilized the gene targeting technology to generate mouse lines with genetic deficiency of CGT (Bosio et al., 1996; Coetzee et al., 1996, 1998; Stoffel and Bosio, 1997; Suzuki et al., 1998).

It is known that CGT synthesizes not only galactosylceramide but also psychosine (galactosylsphingosine); (Morell and Radin, 1969), monogalactosyl-diglyceride (Van der Bijl et al., 1996), and the precursor of seminolipid, 1-alkyl, 2-acyl, 3-galactosyl-glycerol (unpublished). Galactosylceramidase in turn also hydrolyzes these additional CGT products. No CGT product is known to date that is also not a substrate of galactosylceramidase. This would lead to a logical prediction that, if both CGT and galactosylceramidase are genetically deficient, the resultant phenotype should be only that of CGT deficiency because lack of degrading enzyme should be of no consequence, when the synthetic machinery is absent. The availability of the twitcher mouse and the experimentally generated CGT knockout mouse provides a unique opportunity to test this hypothesis, since they allow generation of a mouse line genetically deficient in both enzymes. This report describes the outcome of such an experiment, which indicates that the logical prediction was only partially correct and that there are aspects of sphingolipid metabolism related to CGT and galactosylceramidase that are yet to be clarified.

MATERIALS AND METHODS

Commercial Materials

AmpliTaq DNA polymerase was purchased from Perkin Elmer Corp. (Roche, Branchburg, NJ). Restriction enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN). NuSieve 3:1 agarose gel was from FMC BioProducts (Rockland, ME). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and other standard suppliers.

Generation of Mice Doubly Deficient in Galactosylceramidase and CGT

Twitcher mice (*galc* $^{-/-}$) were on the C57BL/6J background. Our *cgt* $^{-/-}$ mice had exon 2 of the *cgt* gene disrupted with the neomycin-resistance (*neo*) gene (Coetzee et al., 1996). The line was back-crossed to the C57BL/6J background for several generations to minimize the potential complications due to cross-breeding between two lines of different genetic background. Heterozygotes of CGT-deficient mice (*cgt* $^{+/-}$) were mated with *galc* $^{+/-}$ mice to generate double heterozygous mice (*galc* $^{+/-}$, *cgt* $^{+/-}$). The double carriers were mated to obtain double knockout mice (*galc* $^{-/-}$, *cgt* $^{-/-}$) at a probability of 1/16.

Genotype Determination by Polymerase Chain Reaction (PCR)

Genotypes of the hybrid mice were determined by appropriate PCR procedures. Genomic DNA was extracted from a clipped tail according to the standard procedure and digested with proteinase K at 55°C overnight. To diagnose the twitcher mutation, a pair of mismatched PCR primers were designed around the mutation at codon 339 in such a way that the resultant PCR product from the wild-type allele is digested by Hpa II (91 bp and 17 bp), while that from the twitcher allele is resistant to Hpa II (Matsumoto et al., 1997). The primers were: 5'-AGTTTACTCAACCAGGCCG-3'(sense) and 5'-CAATGATGATGGTGAGGTTTCCC-3'(anti-sense). The PCR reaction was performed in 50-μl volumes containing 5 μl of ×10 PCR buffer (15 mM $MgCl_2$), 1.0 μM of each primer, 0.2 mM of dNTPs, 2 units of AmpliTaq DNA polymerase and 100–200 ng of genomic DNA. Samples were denatured for one cycle at 94°C for 2 minutes, followed by 30 cycles at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute. The amplified fragment was digested by Hpa II at 37°C and the sizes of the final products were evaluated by electrophoresis in 4% NuSieve 3:1 agarose gel.

From time to time, the PCR diagnostic procedure originally described by Sakai et al. (1996) was also used for diagnosis of the twitcher mutation. Sakai et al. (1996) did not specify the reaction mixture. The following condition was optimal in our hands. The PCR reaction was performed in 50-μl volumes containing 0.4 μM of each primer, 0.2 mM of dNTPs, 1.5 units of Taq DNA polymerase, and 100–200 ng of genomic DNA in 60 mM Tris-HCl, pH 9.5, 15 mM ammonium sulfate, 3.5 mM $MgCl_2$, and 4% dimethylsulfoxide. Samples were denatured for one cycle at 94°C for 2 minutes, followed by 35 cycles at 94° C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute, and the final extension reaction at 72°C for 7 min.

To determine the status of the *cgt* gene, both the Southern blotting procedure described earlier (Coetzee et al., 1996) and a more recently devised PCR procedure were employed. For PCR, two pairs of primers were used. One pair of primers was designed to detect intact exon 2 of the mouse *cgt* gene. The primers were: 5'-CTCTCAGAAGGCAGAGACATTGCC-3'(sense) and 5'-CATCCATAGGCTGGACCCATGAAC (antisense).

The other set of primers was designed to detect presence of the *neo* gene. The primers were:

5'-GGAGAGGCTATTCGGCTATGAC-3'(sense) and
5'-CGCATTGCATCAGCCCATGATGG-3' (antisense).

Amplification was done with these two pairs of primers present simultaneously in the reaction mixture. PCR was performed in 50-μl volumes containing 5 μl of ×10 buffer, 0.5 μM of each primer, 0.2 mM of dNTPs, 2.5 units of AmpliTaq DNA polymerase, and 100–200 ng of genomic DNA. Sample were denatured for one cycle at 94°C for 2 minutes, followed by 30 cycles at 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 1 minute, and the final cycle at 72°C for 10 minutes. The first set of the primers generated 558-bp fragment from intact exon 2. The second pair of the primers amplified a 315-bp segment of the *neo* gene when present. Thus, wild-type mice gave only the 558-bp band, *cgt* $^{-/-}$ mice gave only the 315-bp band, and *cgt* $^{+/-}$ mice both bands. All of these procedures combined allowed unequivocal determination of the genotypes of both the *galc* and *cgt* genes.

Clinical Studies

All mice were closely observed throughout their lives. Genotypes were determined before 10 days after birth. Body weight was recorded daily as one of the objective measures for

development and progression of the disease. In order to determine the natural course of the disease, some mice were allowed to live as long as they could be maintained humanely according to the acceptable practice of laboratory animal care but without forced feeding, intubation, and other extraneous interventions. For pathological and biochemical examinations, specimens were obtained at 20, 35, and 43 days for three genotypes: — double knockout (*galc* $^{-/-}$, *cgt* $^{-/-}$), twitcher (*galc* $^{-/-}$, *cgt* $^{+/+}$), and the wild-type. For biochemical analyses, the brain and kidney were carefully dissected out and kept frozen at -80°C until analysis.

Pathology

Mice were anesthetized with ether and perfused through cardiac puncture with phosphate-buffered 4% paraformaldehyde (pH 7.4) or 2.5% glutaraldehyde and immersed at 4°C overnight in the same fixative. Then the brain, optic nerve, spinal cord, trigeminal nerves, spinal ganglia, and sciatic nerves were removed. Tissues were processed, embedded, sectioned, and examined by light and electron microscopy and immunocytochemistry (Oya et al., 1998).

Lipid Analysis

The whole brain was homogenized in four volumes of distilled water in a Potter-Elvehjem glass homogenizer with a glass pestle. The procedure for the general lipid analysis was for the most part as we described earlier (Fujita et al., 1996). Brain psychosine (galactosylsphingosine) was determined by the high-performance liquid chromatography (HPLC) procedure (Merrill et al., 1988; Rodriguez-Lafrasse et al., 1994) as modified by us more recently (Matsumoto et al., 1997), except that the mobile phase was methanol-5 mM sodium phosphate buffer, pH 7.0 (89:11) with 50 mg/ml sodium octylsulfate as a ion-pairing agent. The peaks of phytosphingosine, psychosine (galactosylsphingosine), sphingosine, sphinganine, and eicosasphinganine (internal standard) were eluted in this order and were cleanly separated from each other and from other interfering fluorescent materials. Glucosylsphingosine and galactosylsphingosine were eluted with the same retention time in this HPLC system. The tissue levels of psychosine corrected for the internal standard and the relative detector response were expressed in pmol/mg tissue protein.

RESULTS

Clinical Observations

Mice doubly deficient with galactosylceramidase and CGT (*galc* $^{-/-}$, *cgt* $^{-/-}$) could not be distinguished clinically from normal littermates up to postnatal days 10–12. Then, double KO mice began to exhibit gross jerking of the head and mildly ataxic locomotion. The age of the clinical onset was about 12 days compared to 18–20 days in twitcher mice with the normal *cgt* background (*galc* $^{-/-}$, *cgt* $^{+/+}$) and was similar to CGT knockout mice (*galc* $^{+/+}$, *cgt* $^{-/-}$). At around 14 days, tremors became prominent even at rest and very pronounced during movement. Gait was unstable, characterized by splaying of the hind limbs, dragging of the hindquarters as if crawling. While difficult to describe in words, these symptoms were identical with those of *cgt* $^{-/-}$ mice but clearly different from those of twitcher mice. Thus, the earlier clinical phenotype of the double knockout mice was, as expected, that of the CGT knockout mice.

However, after 45 days, the double KO mice began to lose weight rapidly. They showed progressive muscle wasting of the hind limbs and a kyphotic posture. Progressive fine and rapid tremor appeared in the head and neck. The physical and neurological deterioration was more rapid than that seen in CGT knockout mice. The nature of these symptoms was also different from that of *cgt* $^{-/-}$ mice but similar to the clinical phenotype of twitcher mice in their later stages. The overall survival of the doubly deficient mice was unlike either twitcher mice or CGT knockout mice. Similar to *cgt* $^{-/-}$ mice, about 50% of the doubly deficient mice died between 20 and 30 days. However, the remaining double knockout mice had much shorter life span than *cgt* $^{-/-}$ mice. When the mice that survived for 45 days or longer are compared, the double knockout mice died around 60 days on the average, while CGT knockout mice lived much longer up to 99 days on the average (Figs. 1 and 2). The maximum body weight attained was about half of wild-type littermates, lighter than that of *cgt* $^{-/-}$ mice but heavier than that of twitcher mice. (Fig. 3).

In summary, mice doubly deficient in galactosylceramidase and CGT showed a clinical phenotype indistinguishable from that of CGT knockout mice with normal genetic background up to 45 days, but then deteriorated much more rapidly than the CGT knockout mice. In later stages, they exhibited late twitcher-like clinical manifestations.

Pathology

In the animals younger than 43 days, the neuropathology observed in *galc/cgt* double knockout mice was essentially identical with that of CGT knockout mice (Coetzee et al., 1996). Myelination appeared to progress normally until 20–30 days when vacuolation became apparent in the cerebellar white matter, brainstem, and spinal white matter. The vacuolation in the white matter progressed to the point of causing myelin breakdown. In mice older than 43 days, vacuolation was also present in the thalamus, basal ganglia, and spinal gray matter. In 60-day-old *galc* $^{-/-}$, *cgt* $^{-/-}$ mice, many naked or thinly myelinated axons predominated in the white matter. Neuropathologists were under an impression but without objective documentation that vacuolation was more prominent in the *galc/cgt* double knockout than in *cgt* $^{-/-}$ mice with the normal *galc* genotype. Axonal spheroids were found in the internal capsule, cerebellar white matter, brainstem fiber tracts, and spinal white matter in association with myelin degeneration at 60 days. Activation of microglia and astrocytes were noted in the cerebral cortex and posterior thalamic regions. These findings were all similar to those in the *cgt* knockout mouse. Globoid cells, the hallmark of globoid cell leukodystrophy and thus of the twitcher mutant, were never seen at any age. These findings are consistent with the prediction that the phenotype of the double knockout mice should be that of the CGT knock-

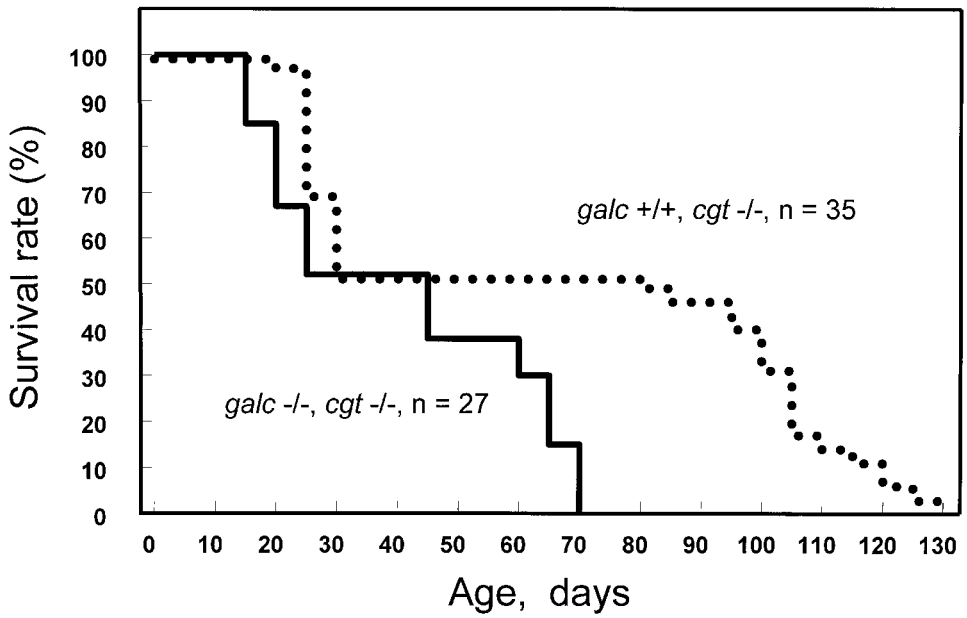

Fig. 1. Life span of the doubly deficient (*galc* $^{-/-}$, *cgt* $^{-/-}$) mice compared to that of CGT knockout (*galc* $^{+/+}$, *cgt* $^{-/-}$) mice. While the survival curve during the early stages are similar, the doubly deficient mice have much shorter life span than the *cgt* $^{-/-}$ mice with normal *galc* genes. Almost all regular twitcher mice (*galc* $^{-/-}$, *cgt* $^{+/+}$) die by 55 days.

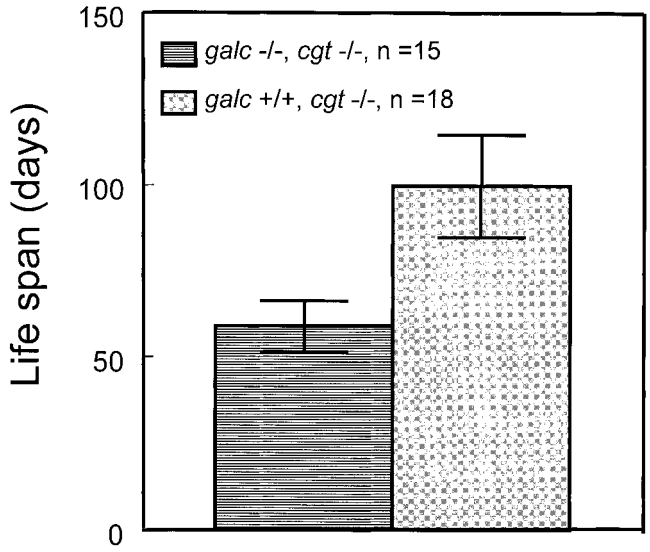

Fig. 2. Life span of the doubly deficient (*galc* $^{-/-}$, *cgt* $^{-/-}$) mice compared to that of CGT knockout (*galc* $^{+/+}$, *cgt* $^{-/-}$) mice. Only those mice that survived for 45 days or longer are compared in this graph. The statistical scatters are S.D.

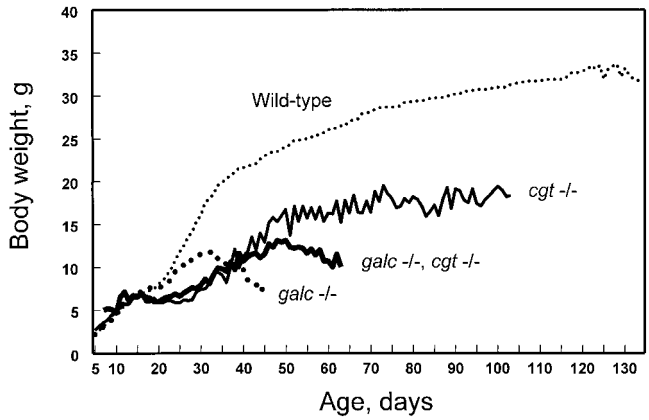

Fig. 3. Body weight of mice of different genotypes. CGT knockout mice (*cgt* $^{-/-}$) develop the disease earlier than twitcher mice (*galc* $^{-/-}$) and consequently start losing weight earlier. However, twitcher mice deteriorate much more rapidly once the disease process is underway. In this regard, the doubly deficient mice (*galc* $^{-/-}$, *cgt* $^{-/-}$) are similar to the CGT knockout mice up to 45 days but then they deteriorate more rapidly.

out, because the deficiency of the degradative enzyme should be of no consequence if the substrate is not synthesized.

However, this logical prediction did not hold after 45 days. Additional progressive neuronal pathology appeared in the brainstem and spinal cord of the double knockout mice that is not seen in CGT knockout mice. This pathology consisted of ubiquitin-positive intracytoplasmic inclusions mostly in the neurons in the brainstem and spinal cord (Figs. 4, 5). These inclusions stained strongly with toluidine blue on 1-μm-thick sections and were homogeneously electron-dense. They were similar to those seen in some long-surviving twitcher mice originally describe by Duchen et al (1980) and also in long-surviving twitcher mice that had received bone marrow transplantation treatment (Suzuki et al., unpublished; Fig. 5).

The peripheral nerves were well myelinated without any axonal or myelin pathology up to 43 days. However, by 60 days, the double knockout mice developed severe axonal degeneration in the motor segment of the trigeminal nerve (Fig. 6). This pathology was present in at least

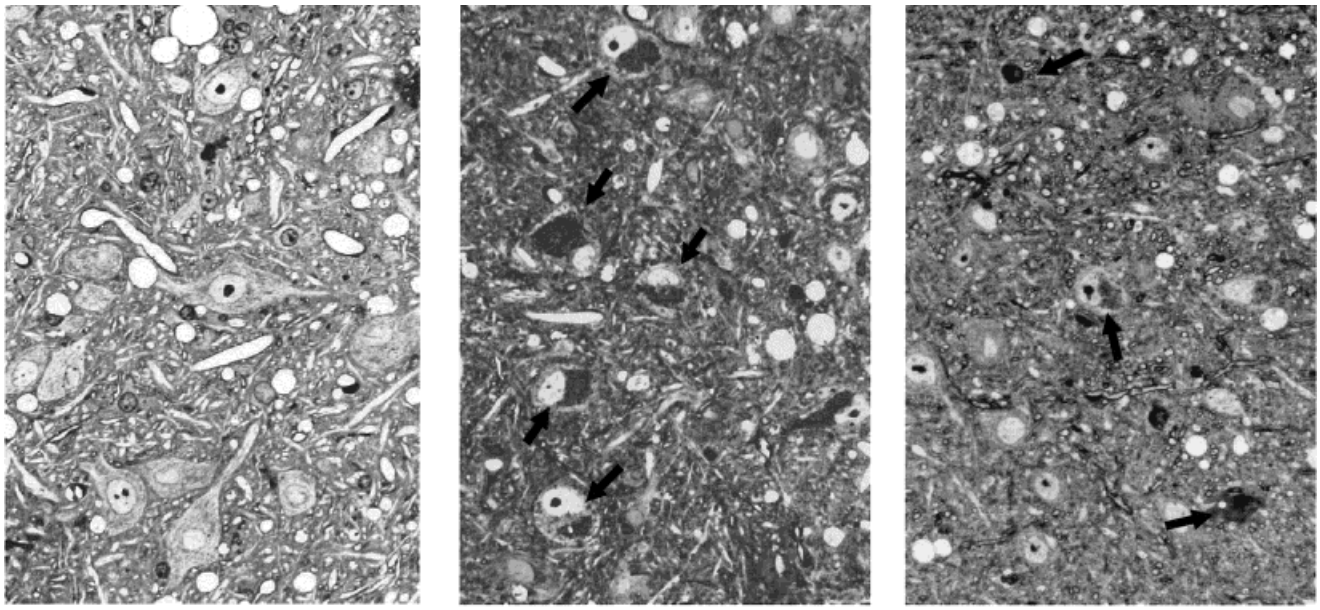

Fig. 4. Late neuronal pathology in doubly deficient mice. After 45 days, doubly deficient mice develop neuronal degeneration (**middle** panel), which is similar to that seen in long-living twitcher mice that received normal bone marrow transplantation (BMT) during the first 10 days of life (**right** panel). Arrows point to examples of degenerating neurons. CGT knockout mice do not show this late neuronal pathology (**left** panel).

three double knockout mice examined in detail. This abnormality appears to be unique in the double knockout mice, not seen either in twitcher mice or in CGT knockout mice.

Biochemistry

Three brain samples were analyzed each at 20, 30, and 43 days. In addition, single samples at 61 and 63 days were included. Brain lipid analysis did not distinguish the double knockout mice from the CGT knockout mice with the normal pairs of the *galc* gene, including those minor constituents metabolically related to galactosylceramide, such as ceramide and lactosylceramide. At the sensitivity of our standard analytical procedure, galactosylceramide was undetectable, partially replaced by glucosylceramide with primarily α-hydroxylated fatty acids (data not shown; Bosio et al., 1998; Coetzee et al., 1996). Galactosylsphingosine (psychosine), which is also synthesized by CGT and accumulates to very high levels in twitcher mice with normal CGT, was also completely undetectable at any age examined (Fig. 7). Galactosylsphingosine is undetectable in normal mouse brain at 15 day but then becomes detectable at 25 days, and the HPLC peak is slightly larger at 43 days. Still, the level of psychosine in normal mouse brain is in the range of 10 pmoles/mg protein at the maximum estimate (Fig. 7). In contrast, psychosine levels in twitcher brain can be a few hundred pmol/mg protein (Fig. 7). Glucosylsphingosine (glucopsychosine) eluted at the same location as galactosylsphingosine in our HPLC system and our data indicated clearly that glucosylsphingosine is essentially absent in normal mouse brain as well as in CGT knockout or twitcher/CGT double knockout mouse brain.

DISCUSSION

The existence of the naturally occurring twitcher mutant due to genetic galactosylceramidase deficiency and the artificially generated CGT (UDP-galactose:ceramide galactosyltransferase)-deficient mouse provided a unique opportunity to dissect out genetically the metabolic relationship of the enzymatic machinery and involved substrates associated with these synthetic and degradative pathways. The advantage of this approach is to avoid complications inevitably associated with other approaches, such as use of exogenous metabolic inhibitors, because the manipulation is to the specific genes on the genomic level. It is pragmatically impossible to ascertain all possible side effects of exogenous agents.

The cross-breeding experiment described in this report was undertaken to answer the question as to whether or not the degradative enzyme is required if the substrate is not synthesized. This question is not as simple-minded as it may sound for two complicating factors: (1) there are other substrates of these enzymes, some perhaps still un-

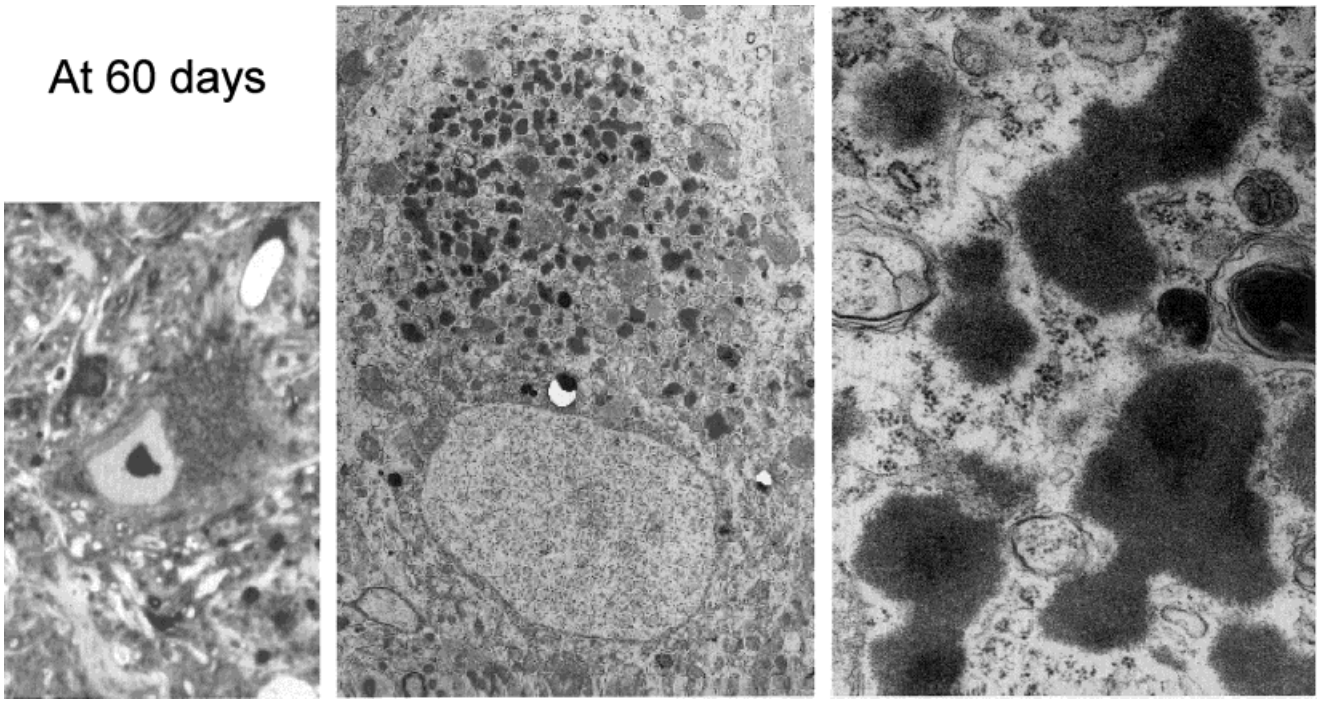

Fig. 5. Ultrastructure of the late neuronal pathology in the brainstem in the doubly deficient mice. The **left** panel shows the neuronal inclusions in toluidine blue-stained 1-μm section. The **center** panel shows a low magnification electron micrograph of a neuron containing numerous electron-dense inclusions in the perikaryal cytoplasm. The **right** panel shows a higher magnification of the inclusions, composed of homogeneous electron-dense material without well-defined internal structures.

known, and (2) although CGT is definitely the primary enzyme to synthesize galactosylceramide, we cannot yet exclude the possibility that some other galactosyltransferases, such as lactosylceramide synthase, which is clearly distinct from CGT, might have minuscule capacity to galactosylate ceramide. CGT synthesizes, in addition to galactosylceramide, galactosylsphingosine (psychosine); (Morell and Radin, 1969), monogalactosyl-diglyceride (Van der Bijl et al., 1996), and very likely also the seminolipid precursor, 1-alkyl, 2-acyl, 3-galactosylglycerol. Galactosylceramide, psychosine, and monogalactosyl-diglyceride are degraded by galactosylceramidase (Bowen and Radin, 1969; Miyatake and Suzuki, 1972; Wenger et al., 1973), and again by analogy, the last is likely to be also degraded by galactosylceramidase. However, there may well be other minor tissue components that are either synthesized and/or degraded by these enzymes. If the one-to-one relationship in synthesis and degradation does not hold for any of such compounds, there can be unforeseen consequences of the double deficiency. On the other hand, if even a minute amount of galactosylceramide is synthesized in the absence of CGT, it can accumulate abnormally in the absence of galactosylceramidase. For example, one such candidate, lactosylceramide synthase, has been purified, cloned, and characterized (Nomura et al., 1998; Takizawa et al., 1999). The published data indicated no acceptor activity of ceramide (Nomura et al., 1998). However, we understand that the data were below the reliable detectability of the standard assay method and that the possibility of exceedingly small activity to transfer galactose to ceramide cannot yet be ruled out (Dr. Tomoko Nomura, personal communication).

The outcome of this series of experiments indicated that the simplistic prediction that mice that lack both CGT and galactosylceramidase should exhibit only the phenotype of the CGT knockout mouse without the phenotype of the twitcher mouse is only partially correct. Up to approximately 40–45 days, the doubly deficient mice were difficult to distinguish from CGT knockout mice without galactosylceramidase abnormality in their clinical and pathological phenotype. However, clinical deterioration was much more precipitous beyond this age and the eventual life span of the doubly deficient mice was approximately 40 days shorter than the CGT knockout mice with normal galactosylceramidase background. Neuronal pathology, which was not present in CGT knockout mice, was observed in these mice. While ordinary twitcher mice die earlier and thus cannot easily be compared with the doubly deficient mice, similar neuronal pathology has been described in twitcher mice that survived for an unusually long period. The twitcher mice originally described by Duchen et al. (1980) had a longer life span,

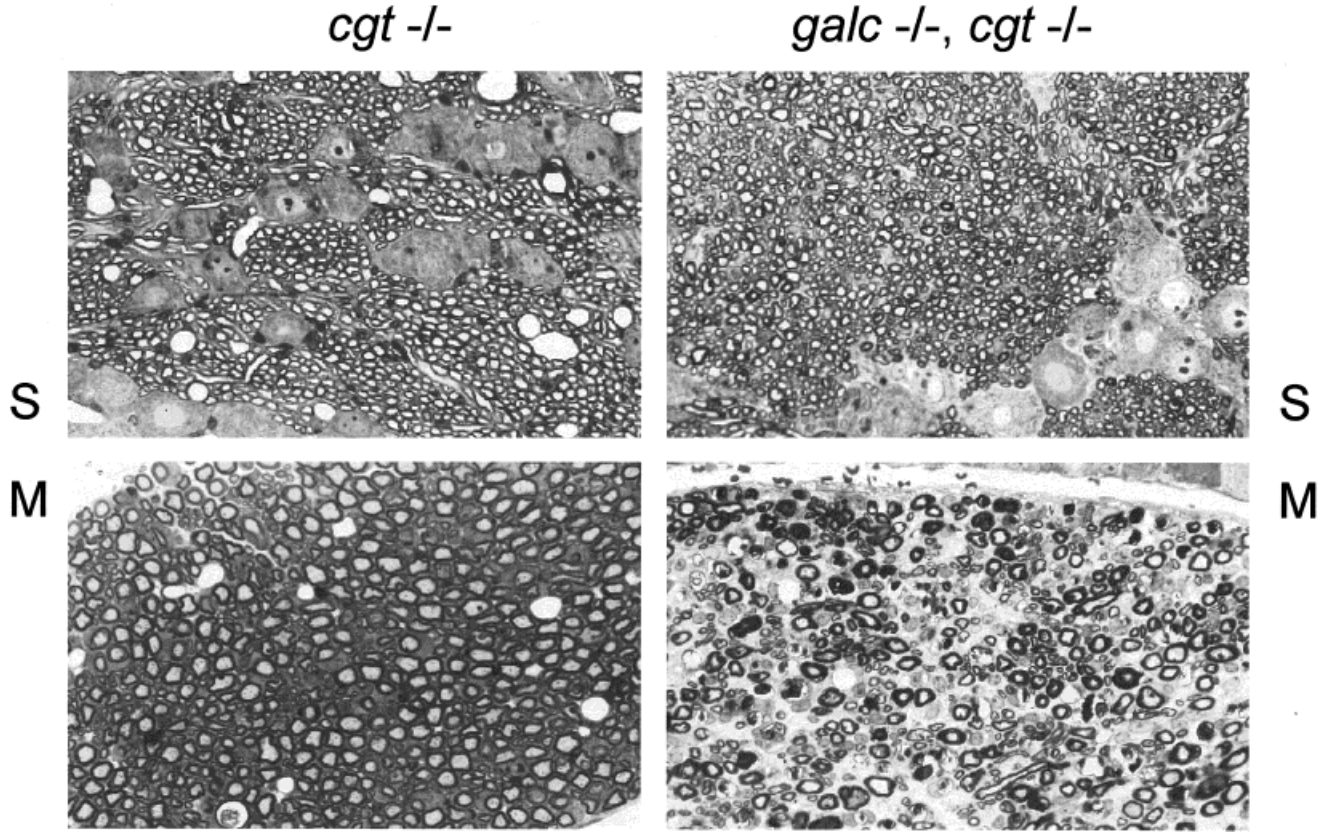

Fig. 6. Severe degeneration of the motor segment of the trigeminal nerve of the doubly deficient mouse. **Left** panels are from a *galc* $^{+/+}$, *cgt* $^{-/-}$ mouse, and **right** panels are from a *galc* $^{-/-}$, *cgt* $^{-/-}$ mouse. Upper panels are the sensory segment (S) and the lower panels are the motor segment (M) of the nerve. The peripheral nerve of *cgt* $^{-/-}$ mice is essentially normal and so is the sensory segment of the doubly deficient mouse. However, the motor segment of the doubly deficient mouse shows severe degeneration, not seen in any other genotypes.

probably due to a different genetic background and they observed late degenerative changes in the cortical neurons similar to those seen in our doubly deficient mice. Similarly, twitcher mice that received bone marrow transplantation treatment before 10 days and survived up to 100 days also exhibited similar degenerative neuronal pathology. These inclusions differ from any of inclusions described in other sphingolipidoses, although they have superficial resemblance to those described in infantile neuronal ceroid lipofuscinosis (Haltia et al., 1973). Furthermore, the degeneration of the motor segment of the trigeminal nerve in the *galc* $^{-/-}$, *cgt* $^{-/-}$ mice has not been described to our knowledge in either twitcher mice or CGT knockout mice.

We interpret these observations to mean that, while the lack of galactosylceramide synthesis by the myelinating cells abolishes the overwhelming twitcher phenotype due to the abnormalities in the myelin and myelinating cells, the pathology that is seen in very late stages of twitcher mice appears to contribute to the much shorter life span of the *galc* $^{-/-}$, *cgt* $^{-/-}$ mice than that of the CGT knockout mice. The metabolic mechanism underlying this phenomenon is not clear. The results of the biochemical analysis were basically identical with those from the CGT knockout mice: complete absence of galactosylceramide and psychosine. Even in later ages, no additional abnormalities were detected. We can therefore only speculate on some of the possibilities. If the metabolic mechanism underlying the late pathology is indeed the same as seen in long-surviving twitcher mice, it suggests that either some unknown substrates of galactosylceramidase are synthesized without CGT or some of the usual CGT products can be also synthesized by other enzymatic mechanism. As of this writing, we have no definitive data to support either possibility, except that a highly sensitive analytical procedure that combined monoclonal anti-sulfatide antibody and the electro-spray mass spectrometry has detected exceedingly small quantities of sulfatide in several tissues of the CGT knockout and twitcher/CGT double knockout mice (Dr. Pam Fredman et al., unpublished). The only known metabolic pathway to generate sulfatide is through galactosylceramide as the precursor. We are unable to offer

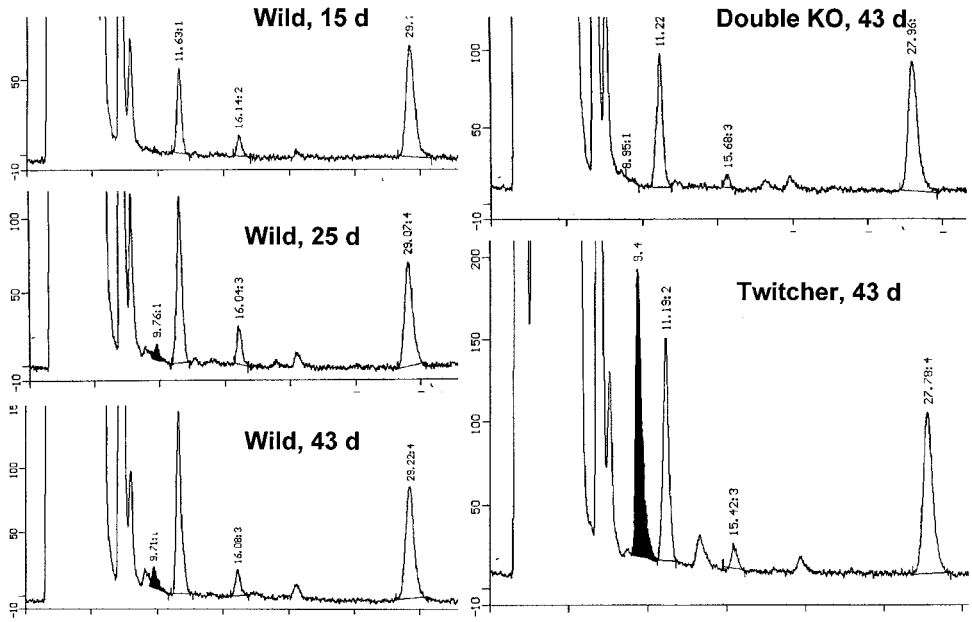

Fig. 7. High-performance liquid chromatography (HPLC) tracings of brain psychosine analysis. The three **left** panels show minuscule but increasing amounts of psychosine in the wild-type mouse brain during development. Twitcher mouse brain has an enormously elevated amount of psychosine (**right lower** panel) but the twitcher mouse with simultaneous complete deficiency of CGT is completely devoid of psychosine in the brain even at 43 days (**right upper** panel). While not shown in this figure, CGT knockout mouse brain gave an identical HPLC pattern as brain of the doubly deficient mouse. Psychosine is shown as a black peak. The peak following psychosine is sphingosine, the peak around 16 min is sphinganine, and the last major peak is eicosa-sphinganine (internal standard). In this HPLC system, glucosylsphingosine elutes at the same location as psychosine (galactosylsphingosine) and therefore this figure also demonstrates complete lack of glucosylsphingosine in the mouse brain. KO = knockout.

even a speculation about the metabolic cause underlying the unusual pathology in the trigeminal nerve.

The results from this series of cross-breeding experiments clearly indicated that the notion, "mice doubly deficient in galactosylceramidase and CGT should give the phenotype of CGT knockout mice because the lack of degrading enzyme should be of no consequence when the substrate is not synthesized" is an oversimplification.

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